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TABLE OF CONTENTS

W. W. Swingle. The determination of sex in animals. *Physiological Reviews*. Vol. 6, January, 1926.

W. W. Swingle and W. F. Wenner. The effect of bleeding upon the serum calcium of thyroparathyroidectomized dogs. *American Journal of Physiology*. Vol. 75, January, 1926.

W. W. Swingle and F. W. Wenner. The prevention and cure of tetany by oral administration of strontium. *American Journal of Physiology*. Vol. 75, January, 1926.

W. M. Copenhaver. Experiments on the development of the heart of Amblystoma punctatum. *Journal of Experimental Zoology*. Vol. 43, February, 1926.

W. Byers Unger. The relationship of rhythms to nutrition and excretion in Paramecium. *Journal of Experimental Zoology*. Vol. 43, February, 1926.

Alexander Petrunkevitch. Spiders from the Virgin Islands. *Transactions of the Connecticut Academy of Arts and Sciences*. Vol. 28, March, 1926.

W. F. Wenner. The prevention and cure of tetany by oral administration of magnesium lactate. *Proceedings of the Society for Experimental Biology and Medicine*. Vol. 23, 1926.

J. William Buchanan. Depression of oxidative metabolism and recovery from dilute potassium cyanide. *The Journal of Experimental Zoology*. Vol. 44, April, 1926.

J. William Buchanan. Some antagonistic and additive effects of anesthetics and potassium cyanide. *Journal of Experimental Zoology*. Vol. 44, April, 1926.

F. H. Swett. On the production of double limbs in amphibians. *Journal of Experimental Zoology*. Vol. 44, April, 1926.

E. Frances Botsford. Studies on the contractile vacuole of Amoeba proteus. *Journal of Experimental Zoology*. Vol. 45, May, 1926.

J. William Buchanan. Regional differences in rate of oxidations in the chick blastoderm as shown by susceptibility to hydrocyanic acid. *The Journal of Experimental Zoology*. Vol. 45, May, 1926.

O. M. Helff. Study on amphibian metamorphosis. *The Journal of Experimental Zoology*. Vol. 45, May, 1926.

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Karl E. Mason. Testicular degeneration in albino rats fed a purified ration. *Journal of Experimental Zoology*. Vol. 45, May, 1926.

Florence Dowden Wood. Autotomy in Arachnida. *Journal of Morphology and Physiology*. Vol. 42, June, 1926.

Alexander Petrunkevitch. Tarantula versus Tarantula-Hawk: A study in instinct. *Journal of Experimental Zoology*. Vol. 45, July, 1926.

Lorande Loss Woodruff. Eleven thousand generations of Paramecium. *Quarterly Review of Biology*. Vol. 1, July, 1926.

Helen Warton Kaan. Experiments on the development of the ear of Amblystoma punctatum. *Journal of Experimental Zoology*. Vol. 46, August, 1926.

Raymond C. Parker. Symbiosis in Paramecium bursaria. *Journal of Experimental Zoology.* Vol. 46, August, 1926.

Gudrun Ruud. The symmetry relations of transplanted limbs in Amblystoma tigrinum. *Journal of Experimental Zoology.* Vol. 46, August, 1926.

Lorande Loss Wooddruff. The versatile Sir John Hill, M. D. *American Naturalist.* Vol. 60, September-October, 1926.

George A. Baitsell and Karl E. Mason. The origin of the fibrous tissue arising in the guinea pig testis as a result of experimental tuberculosis. *Transactions of the twenty-second Annual Meeting of the National Tuberculosis Association,* 1926.

J. William Buchanan. Disintegrative action of KNC on Amblystoma embryos in solutions of different osmotic pressures. *Society for Experimental Biology and Medicine.* Vol. 24, October, 1926.

William Eben Greenleaf. The influence of volume of culture medium and cell proximity on the rate of reproduction of infusoria. *Journal of perimental Zoology.* Vol. 46, October, 1926.

Wesley R. Coe. Sidney Irving Smith and his contributions to zoology. *American Journal of Science.* Vol. 72, November, 1926.

W. W. Swingle and Kirby A. Martin. The effect upon mammalian heat production of feeding thyroid of cold-blooded vertebrates. *Journal of Experimental Zoology.* Vol. 46, November, 1926.

W. W. Swingle. The effect upon amphibian differentiation of feeding Iodofibrin, Iodoedestin and Iodogliadin. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 24, December, 1926.

E. L. Corey. The effect of forcing fluids, upon survival after bilateral epinephrectomy. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 24, December, 1926.

W. W. Swingle. Blood changed following bilateral epinephrectomy in cats. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 24, December, 1926.

W. F. Wenner. The prevention of tetany by the oral administration of ammonium chloride. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 24, December, 1926.

W. W. Swingle and Anna J. Eisenmann. The acid-base equilibrium of epinephrectomized cats. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 24, December, 1926.

Raymond C. Parker. The effect of selection on the rate of reproduction of Paramecium aurelia. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 24, December, 1926.

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THE DETERMINATION OF SEX IN ANIMALS[1]

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The first and most fundamental fact bearing on sex determination brought to light, was a difference in cellular constitution of male and female animals. This fact was known for years before its significance for sex was realized. Henking (1891) described a chromosome-like element in the male germ cells of an insect, which exhibited peculiar behavior during the reduction divisions preliminary to sperm formation. He observed that but half the sperm cells received this body, while the other half were without it. Thus, two classses of spermatozoa are formed. Similar observations on other insects were reported by Paulmier (1899), Sinéty (1901) and McClung (1902). The last named was the first to suggest that the peculiar chromosome was probably concerned with sex determination. Wilson (1905–06) and Stevens (1905) demonstrated that the females of various insects have one more chromosome than the male, and that consequently the diploid chromosome group of this sex in some animals has two X-chromosomes (the term first applied by Wilson, 1909, to the odd chromosome of the male), the male but one. In the course of gamete formation, the eggs are all alike but the sperm are of two classes with respect to the X-chromosome. Thus cytological observation demonstrated a fundamental morphological difference between the sexes in some animals which distinguishes every cell in the organism.

A second important advance, was the correlation of chromosome behavior during gametogenesis with the behavior of the Mendelian factors in heredity (Sutton, 1903; Wilson, 1902), and the assumption (now an established fact) that the chromosomes are the bearers of the hereditary units. For, as we shall see later, the study of sex inheritance has thrown a flood of light upon the problem of sex determination, and the relation of the latter to certain chromosomes. We shall discuss first some of the evidence that sex is dependent upon chromosome constitution.

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[1] The writer takes this opportunity of expressing his obligation to the Biological Laboratory, Cold Spring Harbor, for laboratory facilities during the summer of 1925, when much of this paper was written.

MALE DIGAMETY. This term denotes the condition in which the male forms two kinds of sperm cells and female but one kind of egg. It is by far the commonest type met with among animals and, in fact, is characteristic of most animal groups except the Lepidoptera (moths and butterflies) and birds.

One-half of the sperm cells, in forms exhibiting one type of male digamety, contain an X-chromosome and hence are female-producing under ordinary conditions, while half lack the X and are male-producing. The cells of the female contain one more chromosome than the male and consequently the total chromosome number is even in the female and odd in the male. All of the mature eggs are alike, each receiving a haploid chromosome series including an X, during maturation. The unpaired X-chromosome of the male has no mate, and during gametogenesis enters but half the sperm. The latter thus forms two classes numerically equal, one-half containing an X and the other half lacking this chromosome. The sex of the zygotes is thus seen to depend upon the class of sperm that fertilizes the egg. There should result in each succeeding generation equal numbers of XX (female) and XO (male) individuals. The bisexual condition is thus perpetuated by a chromosome mechanism. It should be reiterated that the condition described is well established by cytological observation and experimental breeding. The two types of sperm, X and no-X, with the chromosome number clearly visible, can be observed in the living spermatozoa of the nematode worm, Ancyracanthus. It has been possible to establish the entrance into the egg of both classes of sperm, and to determine that the sex of the resulting individual depended upon the type of sperm fertilizing the egg. The X-bearing sperm resulted in females, the no-X giving rise to males. This was first demonstrated through the researches of Mülsow (1912).

Besides the type of male digamety in which the female has one more X-chromosome than the male, there is another type in which the chromosome number in the two sexes is the same, but the mate of the X-chromosome in the male differs from it in several features, and consequently is known as a Y-chromosome (Wilson, 1909). The sex formulas thus become XY = male and XX = female. A large number of animals, including man, exhibit this type of digamety. During spermatogenesis in this type, the X and Y of the male join just as do the two X chromosomes of the female, and are then separated at the reduction divisions so that half the sperm receive X and half Y. All of the mature eggs receive a single X. Fertilization of an egg by the X-bearing sperm will produce a female, XX, and if a Y-bearing sperm enters the egg, the male combination XY results. In principle, the XX-XY type is the same as the XX-XO type. In fact, breeding experiments have clearly demonstrated that the Y chromosome lacks sex factors, i.e., it exerts no influence on sex (Bridges, 1914, 1916). Insofar as having any influence on sex is concerned, the Y might just as well be absent, as in the XO type. In fact Wilson (1910) observed that the Y is absent in some specimens of Metapodius.

There are numerous interesting modifications of the XY type (Wilson, 1905; Stevens, 1910; Payne, 1912; Painter, 1922; Schrader, 1922) which occur in various animal groups, but the end result for sex is the same, hence such modifications need not detain us here. In both types of male digamety, the single X-chromosome of the male is derived from the mother. This chromosome, therefore, alternates between the sexes in successive generations.

Morgan (1911) and others demonstrated by breeding tests that the X always passes from the male to the female in the production of females, and from the female to the male in the production of males. We shall see later that this fact has an important bearing upon the mechanism involved in criss-cross inheritance of sex-linked characters.

FEMALE DIGAMETY. Two animal groups, Lepidoptera and birds, present an interesting situation with regard to their chromosomal constitution. For genetic and cytological evidence proves beyond doubt that in the moths and butterflies, the sex chromosome situation is exactly the opposite of that characteristic of the XX-XO (or Y) type. In these insects the eggs are of two kinds, whereas the sperm are all alike, and the phenomenon is known as female digamety. Birds also exhibit this type of digamety as shown by breeding tests (Pearl and Surface, 1910, and numerous others) but to date cytological confirmation is lacking, due to certain technical difficulties inherent in the material. But so far as direct observations go of the chromosome complement of birds, it agrees with the breeding results (Hance, 1924; Shiwago, 1924).

The sex-chromosomes of Lepidoptera were first demonstrated by Doncaster (1910–1915) and Seiler (1914–1922). In the male moth all of the chromosomes are paired, and thus evenly distributed to the sperm so that all of the latter are alike. In females, however, two types of eggs are formed corresponding to the XO-XX type. For example, Seiler found that in Fumea casta the male diploid chromosome number is 62, the female number 61. All the sperm receive 31 chromosomes but the eggs are of two types, one containing 31 chromosomes, the other 30 chromosomes. It is obvious that at fertilization individuals with 62 chromosomes (males) and individuals with 61 chromosomes (females) will occur in equal numbers. The females of certain Lepidoptera are said to be XY instead of XO in constitution.

An interesting situation arises here, in that the sex-chromosome relations in cases of female digamety are exactly the reverse of those in male digamety. It becomes something of a problem to correctly designate the sex-chromosomes. In the Lepidoptera, in as far as the chromosome situation is concerned, XX = ♂ and XO = ♀ but in male digamety it was stated that XX = ♀ and XO (or XY) = ♂, so the symbol X does not mean the same thing in the two types. Because of this fact, cytologists and geneticists have employed a different terminology for the sex-chromosomes in cases of female digamety, and have substituted the letters W and Z for X and Y.

Both types of digamety employ the same general type of chromosome-mechanism to insure the production of sex digamety in each generation. Moreover, the phenomenon of sex-linked inheritance is similar in principle in both types.

We have indicated certain fundamental cytological differences in cellular constitution of the sexes, and expressed the current zoölogical belief that certain chromosomes function in some manner as sex determiners. It will be of interest at this point to present some of the evidence from experimental breeding, that the so-called sex chromosomes are really vitally concerned in sex determination.

Sex-linked inheritance. The evidence from genetics has shown beyond doubt that the sex chromosome carries many other factors than those associated with sex. The behavior in inheritance of characters, the factors for which are carried by the sex chromosome, is peculiar, because they are linked, so to speak, with sex. By this is meant that, in inheritance, those factors which are carried by the sex chromosome are distributed by the same mechanism that determines the normal distribution of the sexes. Study of sex-linked inheritance clearly indicates the importance of the sex chromosome as a sex differentiator (Doncaster, 1908–1914; Morgan, 1910; Pearl and Surface, 1910).

Numerous well-authenticated cases of sex-linked inheritance have been worked out in animals with male and female digamety (Morgan, 1910; Bateson and Punnett, 1911; Goodale, 1911; Sturtevant, 1912; Davenport, 1912). The classical case reported by Doncaster (1908–1914)in the currant moth (Abraxas) will serve as an illustration. It will be recalled that moths exhibit female digamety, i.e., are WZ–ZZ in chromosome constitution.

Doncaster found that in this moth, the wild species (grossulariata), has a variety, known as lacticolor, which differs from the former by having less black pigment on the wings. When a dark grossulariata male is mated to a light lacticolor female, both sons and daughters are dark, i.e., of the grossulariata type. If these are inbred all the sons are dark, whereas half the daughters are dark and half light. The distribution of the Z-chromosome furnishes the mechanism whereby it is possible to explain sex-linked inheritance in this moth.

In the reciprocal cross, lacticolor male by grossulariata female, the daughters are lacticolor like the father, and the sons are grossulariata like their mother (criss-cross inheritance). When the hybrids of such a cross are inbred, the result is lacticolor males and females, and grossulariata males and females in equal numbers.

This type of sex-linked inheritance can be explained on the assumption that the factor for lacticolor is carried by the Z-chromosome. It is evident that in this case the genetic evidence calls for a chromosome mechanism in which the female is heterozygous for sex, since the eggs carrying the grossulariata factor develop into females, the others into males. As stated before, the cytological investigations of Doncaster and Seiler have demonstrated that in Lepidoptera the female produces two kinds of eggs in regard to chromosome constitution, and the male but one kind of sperm. Thus we see that the genetic and cytological findings agree. Many other similar cases are known and the same situation holds true for male digamety, except here the distribution of the sex-linked characters accords with the fact that the male is heterozygous for sex. Occasionally, however, the predicted distribution of sex-linked characters to the offspring does not come out according to expectation, and the theory seems at first sight not to fit the facts. However, study of these exceptional cases has brought to light a unique and most striking demonstration of the relation of sex to chromosomes. The phenomenon is known as nondisjunction.

Non-disjunction. One of the most interesting and suggestive investigations of the mechanism of sex inheritance, and the relation of sex chromosomes to sex distribution, we owe to Bridges (1913–1916). His studies were concerned with the origin of certain exceptional individuals which appeared from time to time in cultures of the fruit fly, Drosophila. Ordinarily, in the case of sex-linked characters, when a female with the recessive character is mated to a male with the dominant character, all the females of the F_1 generation exhibit the dominant sex-linked character, and all the males the recessive character. The reason for this is that the male receives his X-chromosomes from his mother, whereas the father transmits his X-chromosome bearing the dominant allelomorph to his daughters. Bridges studied the inheritance of the factor for vermilion eye-color. The vermilion factor V is borne by the sex-chromosomes, and since the males from crosses between vermilion females and normal red-eyed males receive their only X-chromosome from the mother, they should all be vermilion-eyed. The females from such a cross receive from the father an X-chromosome bearing the dominant allelomorph of V, consequently they should all be red-eyed. In the vast majority of cases this is actually so, but occasionally a vermilion female or a red-eyed male appears as an exception. The study of these exceptional flies led to the discovery of non-disjunction, for their occurrence could only be explained on the assumption that occasionally the two X-chromosomes failed to separate at the reduction divisions during gametogenesis, both passing out of some of the eggs of the vermilion-eyed individual into the polar-body, or conversely, both remaining in the egg. If the two vermilion-bearing X's should remain in the egg then such an egg fertilized by a Y-bearing sperm (Drosophila is of the XX-XY type) would give rise to a vermilion-eyed exceptional daughter. Likewise the no-X egg fertilized by the X-bearing sperm of a red-eyed male would give a red-eyed exceptional male. The cells of the vermilion daughters would contain two X's and a Y-chromosome, thereby differing from the ordinary daughters which contain but two X's. Thus these females possess three sex chromosome (XXY) instead of a pair (XX) and consequently at the reduction division two chromosomes will pass into one cell and one into the other making four classes of eggs possible:

X XY XX Y

If an XXY vermilion daughter is mated to a red-eyed male the progeny produced by the X-bearing sperm would have the following sex chromosome constitution: (Morgan, Sturtevant, Muller, Bridges, 1923)

(1)	(2)	(3)	(4)
XX	XXY	XXX	XY
♀ Red	♀ Red	dies	♂ Red (exception)

The same series of eggs fertilized by the Y-bearing sperm (male producing under ordinary circumstances) gives the following classes of individuals.

(5)	(6)	(7)	(8)
XY	XYY	XXY	YY
♂ ver.	♂ ver.	♀ ver. (exception)	dies

The exceptional vermilion females (7) are of the same chromosome constitution as the original fly and consequently should repeat the process and be non-disjunctional. Breeding tests show that such is actually the case. The red-eyed male (4) should not give exceptional results when mated to normal females. This is obviously so because it has the constitution of normal males.

The females are of two kinds, half (1) should behave like normal females heterozygous for vermilion, and the other half (2) should give exceptions. Repeated breeding tests confirmed the expectations.

The vermilion male (5) and (6) are not alike: one kind (5) is normal, and the other (6) has two Y-chromosomes. Some sperm of the latter would be XY and give daughters which would not themselves be exceptional, but females with a formula XXY should produce exceptions. By breeding experiments, Bridges showed that from half of the males of class 5 and 6 daughters are produced that give non-disjunction.

The genetic results confirm to a nicety the hypothesis that the exceptional individuals are due to non-disjunction of the sex chromosomes caused by the presence of a Y-chromosome in the female. Cytological investigation of non-disjunctional females shows that such females do contain a supernumerary Y-chromosome. As a result of this brilliant investigation it is conclusively demonstrated both by experimental breeding and cytological observation that an abnormal distribution of all the sex-linked characters goes hand in hand with an identical abnormal distribution of sex chromosomes.

L. V. Morgan (1922, 1925) has described another case of complete reversal of the ordinary criss-cross inheritance of recessive sex-linked characters in a line of Drosophila. We saw in discussing the work of Bridges, that in ordinary sex-linked inheritance the recessive sex-linked characters of the mother are transmitted to the sons, while the daughters show the dominant allelomorphic characters of the father. In the example studied by Mrs. Morgan, the reversal was assumed to be due to union of the two X-chromosomes of the mother so that they behave at reduction as a single body (XX-linkage). Cytological examination of such flies showed that the eggs of the females do have two united X-chromosomes. Thus the cytological evidence verified the genetic deduction, and affords further evidence of the relation between sex-chromosomes and the distribution of sex characters.

RELATION OF NON-DISJUNCTION AND XX-LINKAGE TO SEX DETERMINATION. The work of Bridges and Mrs. Morgan is of significance from the standpoint of sex chromosomes and sex-determination. The work of Bridges especially has clearly shown that sex is not predetermined in the gametes but is determined by the chromosome constitution established in the egg at the time of union of the gametes. It was previously stated that the X-bearing sperm in male digamety is the female-determining one, and the Y or no-X sperm the male-determining element under normal conditions. However, in certain cases of non-disjunction these conditions are reversed, and the X-bearing sperm becomes the male determining one, and the Y-bearing sperm the female determining body. This apparent contradiction instead of rendering the argument void, offers the best proof yet obtained that it is not the sperm or egg as a whole that determines sex, but the X-chromosome. The Y-class of sperm, normally male-producing, becomes female-producing if it fertilizes a non-disjunctional egg containing two XX's which have failed to disjoin in oögenesis. Conversely, the X-class of sperm, normally female-producing, becomes male-producing if it enters an egg of the Y class.

The fact stated become apparent if the diagrams of the classes of eggs formed by a non-disjunctional female are studied. These are: egg X, egg XY, egg XX, egg Y. The sperm are of two classes X and Y and the X bearing sperm is female-determining, because the presence of two XX's in an egg always results in females. Thus (Wilson, 1925):

1 Egg X fertilized by sperm X = XX ♀
2 Egg XY fertilized by sperm X = XXY ♀
3 Egg Y fertilized by sperm X = XY ♂
4 Egg XX fertilized by sperm X = ? zygote non-viable

It will be noted that in (3) the egg Y fertilized by an X sperm (normally female producing) results in a male XY in constitution. The same is true when the four types of eggs are fertilized by Y-bearing sperm.

1 Egg X fertilized by sperm Y = XY ♂
2 Egg XY fertilized by sperm Y = XYY ♂
3 Egg Y fertilized by sperm Y = ? zygote non-viable
4 Egg XX fertilized by sperm Y = XXY ♀

In (4) the Y-bearing sperm (male-determining normally), if it enters an egg containing XX, becomes female-producing. The evidence seems conclusive that the sex of the individual depends upon the chromosome combination, no matter how it is established. L. V. Morgan's study of XX-linkage demonstrated the same. As a result of the work mentioned, together with some other, which there is not space to discuss here, zoölogists believe that there is but one kind of X-chromosome and that this in itself is neither male-determining nor female-determining, but stands in such a relation to the developing organism that when it is single (XY) it swings development toward "maleness," and when double (XX) it tips the balance in the direction of "femaleness." In other words, the egg is capable of producing either a female or a male individual depending on whether it contains more or less of X substance. This point of view was clearly expressed by Wilson (1909 and 1911) fifteen years ago, as a result of his cytological studies, and long before non-disjunction and XX-linkage were known. (See also Wilson, 1925.)

In the foregoing section we have attempted to present the cytological and genetic evidence that the sex of an individual is dependent upon its chromosomal constitution. In the three following sections this concept will be applied to the elucidation of some sexual phenomena occurring in animals, which at first sight seem decidedly at variance with the facts just outlined.

PARTHENOGENESIS AND CYCLICAL SEXUALITY. Any theory of sex production or determination must take into consideration haploid and diploid parthenogenesis, so common among certain animal groups. At first thought it may seem that parthenogenetic reproduction, and especially the strange phenomenon of cyclical sexuality, is to prove the rock upon which the chromosome theory of sex is to be wrecked, but such is not the case. Indeed, it is in these exceptional types of sex behavior and production that the theory receives its strongest support.

Among animals which reproduce both by means of fertilized eggs and by parthenogenesis, two general types are known. In one type but one kind of egg is produced which is capable of being fertilized and also of developing parthenogenetically. Animals of the second type produce two kinds of eggs, only one of which is capable of developing parthenogenetically, the other requiring fertilization, or at any rate is capable of being fertilized. The Aphids, Daphnids and Rotifers are the groups in which these types occur. For illustrating the relation of parthenogenesis to sex production and determination, we shall use the aphids, since their chromosome cycle has been worked out so thoroughly by Baehr (1909), Morgan (1909–1912), Stevens (1909–1910) and others.

In some aphids there occurs a succession of parthenogenetic females, and the length of this portion of the cycle, i.e., the number of generations has been shown to depend upon environmental conditions. At the end of the series males and sexual females appear. These females have the double number of chromosomes. The males which appear have one less chromosome than the females (Baehr, 1909; Stevens, 1909–11). Morgan (1909) was able to demonstrate that in the case of the closely related Phylloxera caryaecaulis, a similar reduction in number of chromosomes occurs, and that it is due to extrusion of whole chromosomes into the polar bodies during oögenesis, so that the individuals arising from such eggs (males) had one less chromosome than females. During gametogenesis of the male, one cell gets the unpaired X-chromosome, and this cell after an ordinary division, produces two functional sperm, both alike. The cell lacking the X degenerates. The sexual egg gives off two polar bodies, and then contains the haploid number of chromosomes including one X. If such an egg is fertilized by the functional X-bearing sperm it will give rise the following year to a stem-mother which in turn will be the starting point for a new line of parthenogenetic females.

Morgan's work (1909) on the chromosome relations of the phylloxeran of the hickories is a striking demonstration of the close dependence of sex determination to chromosome constitution in the complicated life cycle of these parthenogenetic forms. The chromosome cycle is as follows: There are eight chromosomes in the species Phylloxera caryaecaulis, and of these four are apparently concerned with sex determination; they are the only ones discussed here. The fertilized egg gives rise to a female called the stem-mother. These females attach themselves to the leaves of the hickory and a gall forms around them, in which they lay their eggs. The eggs extrude a polar body, but the division is not reductional because all eggs have the four sex chromosomes. The eggs develop parthenogenetically into winged migrant females. These are of two kinds: those which lay large eggs and those which lay small eggs. All females which are derived from the same stem-mother lay the same kind of eggs. Thus there are two kinds of stem-mothers with respect to chromosome constitution: the female-producing stem-mothers are XxXx and the male-producing stem-mothers are XxXx′. The migrant female XxXx produces large eggs which throw off a polar body, but do not undergo chromosome reduction. The resulting egg develops without fertilization into a small sexual female. The other type of female lays small eggs in which the large X's and the small X's conjugate. One of each of these chromosome pairs is then thrown out into the polar body so that two types of eggs are produced, Xx and Xx′, and these develop without fertilization into small males. In the sexual females, a true reduction division takes place, so that her single egg is of the chromosome constitution Xx. The males on the other hand produce sperm cells, half of which have none of the sex-chromosomes. Sperm of the latter type degenerate, so that only female producing sperm remain. When these fertilize the sexual egg, the resulting eggs are either XxXx or XxXx′, and give rise to the corresponding type of stem mother as indicated by the symbols. Thus the cycle is completed, and it affords an amazing confirmation of the conclusions regarding the relation of chromosome to sex, based on the condition existing in ordinary forms.

There is one point however which is not entirely clear, and that is the manner in which the male-producing and female-producing stem mothers are differentiated. Some sort of differential division must occur during the maturation process of the eggs from which such individuals arise but the evidence on this point is not so clear as could be desired. Morgan (1912) has described several peculiarities of chromosome behavior in the male-producing eggs, which may indicate that a portion of the X-chromosome is thrown out of the cell during the maturation of the stem-mother's egg. Morgan has assumed that one pair of sex-chromosomes is heterozygous in a factor indicated by priming one the X's.

The case of the bee and certain other Hymenoptera offers contradictions to the usual chromosome mechanism of sex production and determination. Bee eggs which develop parthenogenetically give rise only to males, whereas fertilized eggs develop into females. Cytological investigation shows that the queen bee possesses 32 chromosomes. Sex chromosomes are present, but cannot be distinguished from the other chromosomes. During the process of egg ripening the chromosome number is reduced to 16. If such an egg is fertilized, it develops into a female; if fertilization does not occur the egg develops into a male with the reduced number of chromosomes, i.e., 16 and possessing but one X-chromosome. Cytological study has shown that a non-X type of sperm is not formed. The question arises as to what becomes of the no-X sperm since the X-carrying type will give a female, and only females arise from fertilized eggs. Meves (1907) discovered that no reducing division occurs in the gametogenesis of male bees. The first division is abortive and affects only a portion of the cytoplasm, the second division is more complete and results in two unequal nucleated cells, but one is very small and degenerates. All of the functional sperm are of one kind and contain sixteen chromosomes (Petrunkevitch, 1901; Meves, 1907; Nachtsheim, 1913).

We may justly conclude that parthenogenesis and cyclical sexuality in animals, despite the extreme complication of the life cycles and its manifold types (e.g., there are parthenogenetic eggs which give females without reduction, others which give females after reduction; some which give males with reduction, and others which give both sexes with and without reduction) affords some of the best evidence yet presented for the intimate relation between chromosome constitution and sex. For in all of these different conditions, cytology has shown reasons for believing that different mechanisms are present in the different groups which determine the right combination of the X-chromosomes to produce the resulting sex. It is true that numerous gaps in our knowledge exist, and the reviewer has no desire to minimize them, but the evidence at hand is such that we may feel sure that when our information of parthenogenetic forms is complete, there will be no great surprises.

In many animals showing cyclical sexuality such as Aphids, Rotifers and Daphnids, the transition from parthenogenesis to sexual reproduction can be more or less controlled by regulating certain features of the environment. For instance, Whitney (1914, 1917, 1919) has shown that sexual reproduction in Rotifers can be postponed, or even prevented, by a variety of chemical substances in the culture medium, or it may be hastened by the use of green protozoan food, or by increasing the oxygen content of the water (Shull, 1918). Banta (1918–24) has demonstrated that in Cladocera, temperature, food and excretions in the medium affect the advancement or postponement of the sexual phase.

However, it should be held constantly in mind when dealing with cyclical sexuality that these environmental changes which are thought by many to be sex-determining, are really not sex-determining at all when this term is properly employed. For illustration we may take the case of Rotifers. According to Whitney (1914), parthenogenetic females if fed on a poor diet of Polytoma, continue to produce parthenogenetic females like themselves, and parthenogenetic reproduction continues indefinitely. If, however, such females are fed quantities of the protozoan Euglena, they lay eggs which develop into individuals which if early fertilized become sexual females, but if not fertilized lay small eggs. If these eggs develop without fertilization they become males; thus the same female becomes either a sexual female, or a female that gives rise to males. In other words, as Morgan (1919) has pointed out, the change in food (Polytoma to Euglena) induced the appearance of a kind of female that is either a producer of eggs that become males, or a sexual female should she early meet a male.

It is probable, though not yet demonstrated, that these environmental changes produce their effects by inducing changes in the chromosome mechanism of sex production. Indeed, Morgan (1919), Goldschmidt (1920) and others insist that the rhythm of cyclical sexuality is not a problem of sex determination at all, since the rhythms exhibited by the Rotifers and Daphnids do not determine one or the other sex, but either females or both sexes. In the words of Goldschmidt (1923), "The rhythm is nothing but a period which rules the maturation of the egg (as well as the possibility of parthenogenesis) which then itself automatically determines sex." The most recent discussion of this problem is that of Shull (1925), to which the reader is referred.

Gynandromorphism. In many species of insects, and especially in the Lepidoptera, an anamolous sex situation occasionally arises, where the individual exhibits male characters on one side of the body and female characters on the other. Such individuals are known as gynandromorphs, and the phenomenon has an intimate bearing on the relation of chromosomes to sex.

Gynandromorphs are really mosaics, in which the essential features of the two sexes are sharply delimited from each other in different regions of the body. The mosaic formations vary, sometimes the right and left halves of the body are of different sex, including the gonads, in other cases, the anterior portion of the animal presents the characters of one sex, the posterior part those of the other sex, and still other cases are known where male and female parts (always definite and distinct) are arranged in every conceivable manner.

In the solution of the problem presented by such sexually aberrant individuals, we find again that chromosomal constitution is the essential feature. It was early recognized by investigators of gynandromorphism that irregularities in distribution of the X-chromosomes could account for the sex mosaics. The difficulty lay in proving the assumption.

The most decisive evidence yet presented in the solution of this problem is that of Morgan and Bridges (1919) on the sex mosaics of Drosophila. Their extensive work on sex-linked and other characters of the fruit fly, enabled them to trace certain characters known to be carried by the sex-chromosome, in the gynandromorph, and thereby get a clue as to the chromosomal make-up of such individuals. Sex-linked characters are carried by the X-chromosomes. If gynandromorphism occurs in individuals which are hybrid in respect to one or more of these characters, the male and female parts of the hybrid will indicate the chromosome constitution of such parts. Several such cases were studied. For example: a gynandromorph appeared which carried sex-linked characters, the abdomen and thorax were male, but the head was entirely female. The male parts all showed the usual sex differences, and *also the sex-linked recessive character*, yellow body color. If we recall for a moment that a recessive sex-linked character borne by one X-chromosome cannot appear in the presenc of its normal allelomorph borne on the other sex-chromosome, we have a clue as to the chromosome constitution of the male and female parts. In this case it must be the yellow-bearing X-chromosome that gives the yellow, male, side of the gynandromorph, and since the female characters arise when two X's are present, both must be present in the female parts, which will thus be of normal (wild type) color, because the gene for wild type color is dominant to yellow. Such a gynandromorph must have arisen, therefore, at a very early nuclear division of the cleaving egg, in which one daughter X-chromosome failed to pass into one of the daughter nuclei. Morgan and Bridges were able to demonstrate that both nuclei, the one containing XX, and the one with XO (or Y) constitution, contain chromosomes derived from both parents. This was done by making one of the original parents homozygous for a definite character carried by an autosome. If all of the chromosome of the fertilized egg divided normally except the X-chromosome, the autosomal character should be present in both nuclei and therefore present in male and female parts of the mosaic. This was found to be the case. On the basis of this convincing genetic evidence, Morgan and Bridges advanced the view that the great majority of gynandromorphs result from the accidental elimination of one of the sex chromosomes at some early division of the fertilized egg.

Three other explanations of gynandromorphism have been offered, all based upon the chromosome constitution of the individual. The three theories are: 1, Boveri's partial fertilization theory (Boveri, 1888, 1915); 2, Doncaster's (1914) double nuclear fertilization theory; and 3, Morgan's (1905) supernumerary spermatozoan idea. Suffice it to say that all of these theories are based on the idea that irregularity of chromosome distribution is the cause of gynandromorphism, and all have some cytological basis in fact.

The genetic evidence is conclusive that gynandromorphism in insects is due to a disturbance of the X-chromosome mechanism. However, there are a few authentic cases of gynandromorphic birds which are not so easily explained. Weber (1890) and Poll (1909) have both described finches in which the right side of the body was male, and left female, in respect to plumage character and sex glands. Bond (1913–1914) has described a pheasant which had female plumage on the right side of the body and male plumage on the left. The reproductive glands were a mixture of male and female parts.

The explanations advanced to account for gynandromorphism in insects do not cover these cases satisfactorily, and for this reason, the type of plumage characteristic of female birds is dependent upon hormones emanating from the ovary and circulating in the blood-stream. If a hen is ovariotomized (Goodale, 1916) she develops typical cock plumage after the first molt. On the other hand, if a cock is castrated, he does not change his plumage, but if an ovary be transplanted after castration (Goodale, 1916; Pezard, 1918; Zawadowsky, 1922–1923) he loses the rooster plumage and develops that of the hen. Bilateral gynandromorphism in birds, with respect to plumage, is difficult to explain, because it is obvious that the sex hormones are circulating in all parts of the body. Pezard (1922) has offered a tentative explanation of gynandromorphic birds. He assumes that in such a condition there is a somatic and hormonic abnormality. In normal animals both sides of the bird's body react to the threshold quantity of female hormones, and female plumage is symmetrically developed. If we assume that the threshold quantity is abnormally high for one side (possibly due to differences in chromosome constitution of the cells of that side) and that at the same time only the threshold quantity of female hormone is produced in such a bird, a bilateral gynandromorph, with respect to plumage, will result. The quantity of hormones produced will be able to feminize only that side of the animal which is normal. The abnormal side (with high threshold requirements) will reveal the male plumage.

HERMAPHRODITISM AND THE CHROMOSOMAL HYPOTHESIS OF SEX DETERMINATION. Many animal groups exhibit the phenomenon of hermaphroditism, in which both eggs and sperm are produced by the same individual. There are numerous types and gradations of hermaphroditism, and some of the most curious and interesting sex conditions known occur in monoecious forms.

Certain animals, like the earthworm, possess both male and female sex glands, but these are located in different regions of the individual's body. Both eggs and sperm are ripened but self-fertilization does not occur. In other groups of worms, self-fertilization does occur. Some Nematodes and Molluscs exhibit a type of hermaphrodition in which a male or female animal develops eggs in the testis and sperm in the ovary at certain times, with the result that one or the other sex becomes superfluous and practically disappears. Other Nematodes and Molluscs are first males and function as such, then later become functional females, and several variations of this type are known but they need not detain us here, since for our problem one kind of hermaphrodition will serve as well as another. The problem with which we are faced is to explain how an individual can produce both eggs and sperm, simultaneously or consecutively.

At the outset of our discussion a difficulty arises, for despite the fact that the study of hermaphroditism offers great possibilities for the elucidation of sex determination and differentiation, the field is practically untouched. Our knowledge of hermaphrodition is the least satisfactory of any in the entire field of sex. It is obvious that it offers difficulties for the chromosome hypothesis, especially the morphological form in which the latter is usually considered. As we shall see later, it is necessary to be clear as to just what the chromosomal hypothesis implies when studying hermaphroditism.

But four cases of sex-chromosomes occurring in hermaphroditic animals are known, and two of these are regarded by some writers as doubtful, and the third is an amazing contradiction to the theory. Thus, we have but one clean-cut case of a direct relation between chromosome behavior and sex, in monoecious animals.

The Nematode, Angiostomun nigrovenosum, has two phases in its life-cycle: a free living generation in which the sexes are separate, and a parasitic generation which lives in the frog's lung and is hermaphroditic. Schleip (1911) and Boveri (1911) independently investigated the cytological conditions existing in this worm. The females of the free living forms (non-hermaphroditic), possess twelve chromosomes, and they produce eggs with six chromosomes after reduction. The males have only eleven chromosomes and produce two kinds of sperm, half with six and half with five chromosomes. However, only the class of sperm with six chromosomes is capable of fertilizing the eggs because the cells of the animals of the succeeding generation always have twelve chromosomes. This generation is hermaphroditic and produces both eggs and sperm, the same sex gland giving rise to both. All the eggs at maturation exhibit the reduced number of chromosomes, i.e., six. In the sperm of the male, two X-chromosomes are unpaired, but the remaining autosomes pair to form five bivalents. These are separated at the first division, consequently the sperm each receive five autosomes and one X-chromosome. However, in one cell the X is eventually cast out and lost. The hermaphrodite thus forms two kinds of sperm, half with five and half with six chromosomes. As a result of this mechanism of chromosome distribution it becomes apparent how the male of the free living generation has eleven and the female twelve chromosomes. The chromosome mechanism is fairly clear in this case and the facts fit in with the chromosome theory. However, it should be pointed out that this case is really not typical of hermaphrodition in general, because: 1, the hermaphrodite generation is really a modified female; 2, the species is hermaphroditic for only a portion of its cycle; and 3, it is not clear why the parasitic generation with twelve chromosomes (XX) should not be a true female instead of an hermaphrodite.

Demoll (1912) and Zarnik (1911) have described sex chromosomes in the snail, Helix pomata, and the pteropod mollusc, Creseis, both of which are hermaphrodites. Zarnik's description of the chromosome-cycle in Creseis is remarkable. The animal possesses twenty chromosomes. In the spermatogonia there are sixteen autosomes, two large X-chromosomes and two small X's. The first division is reductional, resulting in two secondary spermatocytes each with eight autosomes, and one large and one small X-chromosome. At the second or equational division, the small X does not divide but passes intact into one sperm mother cell. Thus two kinds of sperm are formed, one with eight autosomes and one X, the other with eight autosomes and two X-chromosomes. The sperm possessing but one X degenerate. In the oögonia there are sixteen autosomes and four small sex-chromosomes. Following reduction all eggs are alike and contain eight autosomes and two small sex-chromosomes. Fertilization always results in an hermaphroditic individual with sixteen autosomes, two large and two small X-chromosomes.

The case seems clear enough at first sight but unfortunately when closely scrutinized presents several difficulties. The same is true of Demoll's description of the sex-chromosomes of Helix. Neither of these investigations presents us with clean-cut evidence similar to that of Schleip and Boveri.

The fourth case of sex-chromosomes described in an hermaphrodite (?) is Junker's (1923) description of the chromosomes of Perla marginata, a pseudoneuropteran. The sexes are separate in this form and the females present nothing unusual. The males, however, possess an ovarial-like structure situated between the two testes, anteriorly, which consists of egg tubes containing rudimentary and non-functional egg-like cells. The diploid chromosome number in spermatogenesis is twenty-two of which twenty are arranged in ten pairs, while the two large dissimilar elements remain unpaired. They are the two X-chromosomes, X and X′. Two kinds of sperm are formed, half possessing twelve chromosomes and half with ten chromosomes. The diploid number in the female is twenty-four. All the mature eggs are alike and contain twelve chromosomes. Now the peculiar thing about this case is that the cells of the "male ovary," i.e., the ovary-like body in the male, have twenty-two chromosomes, twenty of which are paired, and two unpaired X's. That is to say, the male number of chromosomes is present in the egg-like cells. These cells behave like egg cells except that development is soon arrested and they degenerate. Here we have the astonishing condition of oöcyte-like cells, and a structure closely resembling an ovary, produced in the presence of the chromosome number characteristic of the male—a condition just the reverse of that described for Angiostomum.

There are several significant facts to bear in mind before accepting Junker's radical conclusion that chromosomes have nothing to do with sex determination. Some of the points are:

1. It is significant that the ovarial-like structure is confined to the male.

2. The so called oöcytes or "male-eggs" never become functional but invariably degenerate in early developmental stages.

3. The evidence is far from clear that the structure under discussion is really ovarial, and that the oöcyte-like cells are really immature ova. There are several cases on record where the male sex cells during gametogenesis take on the appearance of egg cells although eventually giving rise to spermatozoa. This is notably true of certain Chilopoda, e.g. Scolopendra and Lithobius (Blackman, 1905; Swingle, 1922).

4. Even if the structure in Perla is a rudimentary ovary, with oöcytes containing the male complement of chromosomes, it is not necessary, in order to account for the phenomenon, to throw overboard the vast amount of convincing evidence that has accumulated showing the determinative effect of chromosome constitution upon sex. It is obvious that there is something lacking in the make-up of the oöcyte-like cells, because they always degenerate. The probable explanation of this

case is that there is some disturbance of the normal balance between the sex factors in the males whereby the ovarial-like structure arises. We shall see later that the sex of cases like Perla is just as dependent upon chromosome and genetic constitution as any other.

The whole subject of the chromosomes of hermaphrodites evidently calls for further investigation. Wilson (1925) has pointed out it should be kept in mind in such an inquiry, that the true hermaphrodite should not be considered as simply a mixture of the two sexes but rather as the result of a definite genetic complex, analogous to that by which the pigment patterns are determined in somatic characters. It may well be that the sex-producing mechanism is cytologically unrecognizable, though present, in such cases. This is certainly true of pigment patterns.

Let us enlarge upon Wilson's suggestion for a moment, and discuss briefly the genetic basis which may lie at the root of hermaphroditism in animals.

How can hermaphroditism, the presence of male and female germ cells within the same individual, be reconciled with the idea of definite chromosomal or genetic factors for sex? Hermaphrodites in their way differ as much from dioecious forms as males and females of the latter differ from one another. Aside from the work of Schleip and Boveri, there is slight cytological evidence for the existence of any chromosome mechanism which could distribute chromosomes or allelomorphic sex factors to different parts of a single animal in the same way that the maturation divisions do in the case of bisexual forms. But it seems unreasonable to suppose that sex in hermaphrodites is a hit or miss affair, in no way related to chromosome constitution or genetic make-up, merely because chromosome differences have not been found (and in many forms probably do not exist visibly), and that presumably there is the same set of chromosomes and genes in the sperm-producing cells as there is in the ova. Like relations undoubtedly hold for other parts of animals besides those concerned with egg and sperm production.

For illustration of this fact we may discuss for a moment coat color in guinea pigs. These animals (Castle and Wright, 1916) present a large range of colors and color blends. A single animal may have black, yellow and white spots scattered over the body, and it is well established experimentally that in the guinea pig there are numerous known genetic factors which are responsible for the colors and color patterns. We must suppose that every cell in a spotted guinea pig contains the same genetic factors for hair color, yet some regions of the body are black, some white and some yellow. How can this be? The genetic and physiological evidence shows that in guinea pigs there are genetic factors present which determine the presence or absence of certain color-producing enzymes and their distribution over the body. It is definitely known that there is a basic color-producing enzyme *a*, which, acting alone on chromogen, produces a finely granular pigment which appears yellow. It is unstable. Its intensity of production, and total absence in parts of the body is determined by a considerable number of hereditary factors (genes), some of which are definitely known. There is a second substance, *b*, which may unite with *a* to produce coarsely granular pigments which appear brown, black and sepia. Intensity of production, or absence of *b* in certain portions of the fur are determined by five definitely known and several unknown genetic factors, which give various types of color patterns.

Here we see that the genetic factor for a color can only come to full expression when acting in definite regions of the body and upon the necessary substratum, i.e., the presence of the basic color-producing enzyme.

It is permissible perhaps to draw an analogy between pigment spotting and the production of real hermaphrodites. Take the common earthworm, in one segment a testis develops, in another segment the ovaries appear. It is highly probable that every cell of the earthworm contains the same complement of genes but the full expression of the male and female determining and differentiating genes can only appear in the right internal environment. Other genetic factors determine the presence or absence of certain necessary agents (hormonal or enzymatic) in those segments in which the sex glands arise, and the germ cells thus come to rest in a different environment, genetically controlled, an environment that is absolutely essential for the expression of the sex peculiar to that segment. It is unnecessary to look for a visible difference in the chromosome constitution in spatial hermaphrodites like the earthworm, any more than it is necessary to rely upon such chromosomal differences to explain spotting in a guinea pig.

Chromosomal differences, in forms like the worm, could only be supposed to arise by somatic segregation, i.e., elimination of certain chromosomes at a definite point in the germ tract in each individual during development. No evidence for such a process exists; nor do we believe there is need for such an hypothesis.

If it is possible to assume that certain types of hermaphroditism are due to regional differentiation with respect to the substratum upon which the sex factors interact, how is it possible to explain such astonishing cases as that described by Babor (1898) for Limax maximus? Here the individuals are first female, then hermaphrodite, then male, then again hermaphrodite and finally female. It is not improbable that in forms with maleness alternating with femaleness in the same individual, the length of the cycle and its nature are genetically determined just as other characters of the individual are determined, and are no whit more mysterious. Just as in parthenogenic reproduction the rhythms can be upset by environmental changes, so too environmental changes can lengthen and shorten the cycle of the sexes or prevent one or the other phase from developing. It is obvious that in Limex and other similar forms the hermaphrodite phase, interpolated between the male and female phases, is a period of transition, and represents the diminution of influence of one set of factors and the beginning influence of another set acting upon genotypically identical material—the sex cells.

In this connection, it is interesting to recall that in maize (Emerson, 1924) any one of nine recessive factors is sufficient, even under ordinary conditions of growth, to upset the typical hermaphrodite habit in this species. Presumably the presence of all nine of the dominant allelomorphs of these factors is necessary for the normal expression of the typical hermaphroditic condition.

Shull (1910, 1911) found that in Lychnis a single factor difference is sufficient to change a bisexual organism into an hermaphroditic one, and moreover the change is a reversible one.

To sum up, we may say that although at first sight the sex phenomena exhibited by hermaphrodites may seem to nullify all that we have said regarding chromosome combinations in relation to sex, such nullification is merely apparent and not real. That morphological chromosome differences have been found in but one or two hermaphrodites, and may not (probably do not) exist in most forms, need not disturb us greatly, for why should sex factors be presumed necessarily to influence the size and behavior of chromosomes more than do other genes? We believe that the problem of sex expression in hermaphrodites is no more mysterious or difficult of solution than the problem of the determination and differentiation of any somatic character, and it is as much dependent upon chromosomal and genetic constitution as any somatic character.

We may consider that in typical hermaphrodites the complex of genetic factors for maleness and femaleness exist in such a delicate balance that influences similar to those which are responsible for differentiation of ordinary somatic characters bring about a more or less regular differentiation of sex organs.

It has doubtless become evident to the reader that the present day trend of zoölogical opinion regarding sex is, that in expression and inheritance it is not fundamentally different from any other character. Practically every body character is the resultant of the interaction of numerous factors although a single factor difference may greatly modify the result. Bridges (1921–1925) has advanced the view, based upon cytological and genetic data, that both sexual and non-sexual characters of an individual are the index of the point of balance in effectiveness of a large but unknown number of genes, some of which have a tendency to change development in one direction and others in the opposite. Let us consider for a moment this idea of "genic balance," and its application to sex characters.

Theory of genic balance. While studying the effects of loss of one of the chromosomes of Drosophila, the so-called fourth chromosome, Bridges was struck by the fact that the phenomenon offered striking parallels to those of dioecious sex. For example, flies having but one fourth-chromosome differed in regard to many characters from flies possessing such a chromosome. The individuals were smaller in size, had smaller bristles, hatched later, were pale in body color, were less viable and had blunter wings than flies with the full chromosome complement. Each of these differences corresponded to a character for which the fourth-chromosome was internally unbalanced. So too in the case of sex, individuals with but one X-chromosome, males, present a complex of characters strikingly different from those shown by individuals with two X-chromosomes, female. Each of the differences presented by the haplo-X individual (male) corresponds to a character for which the balance of genes in the X is different from that in the group as a whole. The absence of one X leaves in action an unbalanced set of genes which produces male characters since the Y-chromosome does not carry sex factors, so far as known. Therefore the X-chromosome is a chromosome that is internally unbalanced by an excess of genes that we may call female-producing. The effects of haploidy for an autosome, e.g., fourth, and the effects normally present in dioecious sex, indicate that they have a similar genic base, each being due to differences in the ratio between two aggregates of genes. The peculiarity of the X-chromosome is that it produces its characteristic effect because it contains a preponderance of genes tending to produce the characters that we call female.

Bridges was able to produce striking evidence for his theory by study of cases in which the ratio of X-chromosomes to autosomes had been changed, and as a result of which new sex types arose. Each of the different sex types proved to be the result of a particular combination of chromosomes, and appeared in cultures of Drosophila that were triploid, i.e., that have three X-chromosomes and three of each kind of autosome. The possession of an extra X and at the same time of an extra set of autosomes does not change the individual in its sexual characters from an ordinary female. However, in oögenesis the triploid or 3n group of chromosomes is unstable, and at reduction several combinations are brought about. Normal males of Drosophila are XY in constitution, one-half the sperm carrying X, one-half Y and fertilization of the eggs of 3n females affords unique opportunity for studying the effects of peculiar chromosome combinations. Some of the individuals resulting from eggs of 3n females will have 3X-chromosomes and two sets of autosomes or be of the constitution 3X, 2A others will be 3X, 3A, or 2X, 2A. The combination 2X, 3A is especially interesting in that the zygote develops into an intersexual form, i.e., an animal that is neither male nor female, but a mixture of both sexes. In chromosome constitution the intersexes do not differ from ordinary females except in the possession of an extra set of autosomes, thus showing clearly that the autosomes are concerned in sex determination. Also they are male-determining, since the addition of a set of autosomes causes the female to assume male characteristics.

We can say, then, that in the autosomes there are genes that tend to produce male characters. Conversely the X-chromosome has a net female tendency as shown by the fact that the addition of an X to a male group of chromosomes changes the individual into a female. The net male tendency of a set of autosomes is less than the net female tendency of an X. This is indicated by the fact that in those individuals in which there are two of each, i.e., 2X, 2A, the female genes overbalance the male and the result is a female.

Bridges also found flies with the chromosome constitution X, 3A and 3X, 2A. According to theory, the former individuals should be more male than normal males, and the latter more female than normal females. The animals in fact differed decidedly from normal males and females and were termed super-males and super-females.

We now have arrived at the point where the old formulation that 2X = female is inadequate, for in the intersexes we have individuals with two X-chromosomes yet are not females. The presence of an extra set of autosomes removes them from the female class. The autosomes have an internal preponderance of male-tendency genes. According to Bridges, we may now formulate the sex-relations as follows: "Both sexes are due to the simultaneous action of two opposed sets of genes, one set tending to produce the characters called female, and the other to produce the characters called male. These two sets of genes are not equally effective, for in the complement as a whole, the female-tendency genes outweigh the male-tendency genes and the diploid (or triploid) form is a female. When the relative number of the female tendency genes is lowered by the absence of one X, the male-tendency genes outweighs the female and the result is the normal haplo-X male. When the two sets of genes are acting in a ratio between these two extremes, as is the case in the ratio of 2X:3A, the result is a sex intermediate an intersex."

In a recent paper, L. V. Morgan (1925) has furnished an interesting confirmation of Bridge's theory of intersexes in Drosophila. She studied a strain of flies in which the two X-chromosomes were permanently attached (XX-linkage). Intersexes arose from certain matings, and cytological examination showed that these individuals had the chromosome constitution, XX, 3A and 2X, 3A.

The "genic balance" interpretation of sex, namely, that sex is probably an expression of the interaction of several, probably many, factors located in different chromosomes has received strong support from the work of Emerson (1924) on maize.

The normal plants of this species are hermaphrodites; the termial inflorescences being male, the lateral inflorescences, female. Certain types of maize are known to have occurred as mutations which are entirely female. Four distinct recessive genes are known, the influence of any one of which results in femaleness. Maize which is altogether male is unknown, but there are three types which produce but a few pistillate flowers. Each of these three types is known to be due to one or more recessive factors, each transmitted independently of the other. Emerson also cites cases of two types of maize in which the monoecious habit has been modified to an andromonoecious one, and he states that each of these two types is also due to the influence of one or other of two recessive genes. From this account, it seems that in maize there are nine (probably many more) pairs of genetic factors which influence the expression of sex, and several of these are known to be borne on different chromosomes.

Sturtevant (1921) has described a race of intersexual Drosophila simulans. The intersexes are modified females and they possess two X-chromosomes even in the male-appearing parts. The modification of the female is produced by a recessive mutant gene located in an autosome. This case shows clearly that the sex-determining mechanism, despite the fact that the reaction had proceeded in a definite direction—female, can be thrown out of gear, so to speak, or greatly modified by the action of an autosomal gene. It is evident, from the works cited, that sex expression is the result of many genes, and not simply of an allelomorphic pair.

So far, the discussion has centered around chromosomes and the Mendelian factors borne by them, and has been colored more or less by morphological considerations. However, the topics discussed in the next section require for explanation formulation in physiological terms. The study of intersexes and sex reversal has rendered it imperative that some of our morphological concepts of sex determination be supplemented by physiological ideas.

INTERSEXES AND SEX REVERSAL IN LYMANTRIA. The best known case of intersexuality and sex reversal is that of the gypsy moth, Lymantria, studied by Goldschmidt (1912–1924). This insect has a wide geographical distribution, and the sexes are well marked off from one another in many structural features. When moths from widely different localities are crossed the progeny exhibit peculiar sexual abnormalities. Thus, crosses of male moths from a Japanese race, with females from certain European races, give normal males, but the females are a mixture of male and female characters. They are called intersexes. Further investigation showed that many different races of Lymantria exist which differ in regard to the factor or factors responsible for the production of intersexuality. This is so, because the extent of the assumption of the characters of the opposite sex is definite and typical for a given cross. Males and females of the same race always give normal offspring, but when the male or female of one race is bred with individuals of various other races, all kinds and degrees of intersexuality result—even complete sex reversal. By making appropriate crosses the sexes can be reversed, and the actual process of reversal studied embryologically.

Goldschmidt accepts the chromosome theory of sex and applied it to his Lymantria material in an ingenious manner. It will be recalled that the progeny of all races employed in the crosses are quite normal when males and females of the same race are used. In the right combination the eggs and sperm give only normal sex results; in hybrid combination, however, the same cells give rise to intersexual individuals. The result seems to depend upon a relationship of something in the eggs to something in the sperm. This something in hybrid combination is quite different from the normal. Consequently, a definite quantitative relation exists between the two sorts of sex-determining substance which is normal, any other quantitative relation produces intersexuality. The degree of intersexuality thus depends upon the degree of variation of such quantitative relations from the normal. According to Goldschmidt, each sex contains the factors for both sexes, and which actually does develop depends entirely on the quantitative relationship between the two factors. For illustration, let F denote the factor for femaleness and M the factor for maleness, then the formula for the two sexes will be (F) Mm = female; (F) MM = male. Goldschmidt places brackets around the F to indicate that it is purely maternal in inheritance. The female is heterozygotic in the factor for maleness, which is carried by the X-chromosome. The factor for femaleness carried in the cytoplasm or W-chromosome is handed on equally to every egg, and is purely maternal. The factor F and M act independently with a quantitatively determined power which may be termed the valency. The factor with the greatest valency determines the result. The quantities are, however, not equal so that one M is weaker than one F, thus in the female sex when both are present the M produces no effect. Two M's, however, are stronger than one F and consequently in the male the F is impotent.

Goldschmidt has formulated some interesting physiological ideas regarding the nature of the sex-determining substances and as to how the quantitative effect is to be interpreted. He identifies the sex factors distributed by the chromosome mechanism with enzymes, and the end result of their activity is the production of hormones which control sex differentiation. Thus, according to Goldschmidt, the provisional solution of the problem of intersexuality in Lymantria is as follows: Each fertilized egg possesses male or female determining factors. These factors are enzymes and each is necessary in order to produce the hormones of sex differentiation. In forms exhibiting female digamety, like Lymantria, every egg is identical in regard to the factor for femaleness. In the male, the sex factor is carried by the X-chromosome and it will be present in half of the eggs and in all sperm. The mechanism of sex inheritance which consists in the distribution of two X-chromosomes to the male (female digamety) and one X to the female, is a mechanism which decides at the commencement of development whether n or two n-units of male enzyme will be oppossed to a definite and always equal quantity of female enzyme. These quantities are so graded that the amount of female enzyme is greater than the n of the male enzyme, and so the production of hormones of female differentiation is accelerated in this combination and the development is female. On the other hand, two n of male enzyme is a higher concentration than q of the female, consequently the hormones of male differentiation will be produced more rapidly and a male will result.

Races of moths vary in regard to the absolute quantity of the enzymes and if two such races are crossed which differ in this respect the normal quantitative relation is destroyed, and male enzymes even in one n quantity may be too strong for the female quantity. The result would be an individual which begins development as a female but as a result of the abnormal quantitative relation of the sex enzymes the hormones of sex differentiation are formed too rapidly, an effective amount is accumulated at some point in embryonic differentiation, and the sex reaction is reversed, the individual ending up as a male or markedly intersexual moth.

As an outgrowth of Goldschmidt's experimental work, zoölogists now recognize two types of intersexuality: zygotic and hormonic, the former occurring in insects, the latter in vertebrates, especially birds and mammals. In insects, each cell of the individual possesses all that is necessary for its determination, and thus each is independent of the remainder of the organism. In other words, sex characters and sex differentiation in this group are not dependent upon the presence of a sex gland. This is a well-established fact as shown by castration and transplantation experiments (Oudemans, 1899; Kellog, 1904; Regan, 1909; Meisenheimer, 1919; Kopec, 1913; Steche, 1912; Geyer, 1913, and Kornhauser, 1919). On the other hand, in birds and mammals (Tandler and Grosz, 1913; Lillie, 1917; Lipschutz, 1914; Sand, 1918; Moore, 1919; Steinach, 1913–1920) the sex factors distributed by the chromosomes at fertilization carry sex differentiation only a short way, so to speak, to the point where a definite sex gland is formed. From this time on, the gonad secretes sex differentiating hormones that are essential for complete sex expression. This is also a well-established fact. In the insect, the hormones are produced in every cell of the body, but in birds and mammals the secretions are localized in one central organ—the sex gland. Thus it is that one can remove the sex gland of larval moths without effect upon the sex characters, whereas in birds and mammals gonad removal in early developmental stages leads to profound alteration of the sex characters—indeed, in birds actual sex reversal may result.

Sex reversal in vertebrates. During the past few years several apparently authentic cases of complete sex reversal in vertebrate animals have been reported. Such cases are of considerable importance for sex theory and need to be carefully examined. We shall discuss briefly the best known cases.

It will be recalled that the genetic evidence shows that the chromosome constitution of birds is the WZ-ZZ type, the female producing two kinds of germ cells, whereas the spermatozoa are all alike. Under normal conditions, the sex of these animals is established at fertilization. However, some recent evidence indicates that the type of sex organization and reproductive functioning of birds is not in all cases irrevocably determined by the sex chromosome constitution, as the following case indicates.

Crew (1923) obtained from the owner a three and one-half year old Buff Orpington hen. The owner claimed that the hen previously laid eggs and had hatched young from them before ceasing to lay at about 3 years. According to Crew, the hen first seen by him was normal in appearance, but was developing a tendency to crow. During the next twenty-two months the hen assumed the plumage, copulatory and fighting behavior of a normal cock, and in fact functioned as a normal male by fertilizing two eggs of a virgin Buff Orpington hen and became the father of two young—a male and a female. After the bird died autopsy revealed extensive tuberculous lesions of the viscera. Two normal-appearing testes were found, and also the degenerate remains of an ovary. Riddle (1924) has described a somewhat similar case in pigeons.

The two cases just mentioned involved sex transformation of adult animals. The following case was experimentally induced. Benoit (1923) discovered that following removal of the left ovary of young fowls (the right ovary normally degenerates in embryonic life), the birds assume the characters of normal cocks and the assumption of these characters is correlated with the development of a structure resembling a testis on the site of the degenerate right ovary. Living sperm cells were found in the birds. Previously, Zawadowsky (1922) and Goodale (1916) had noted the formation of a peculiar testis-like body on the right side following ovariotomy, but they failed to recognize its significance.

Benoit regarded his work as demonstrating that female birds are really suppressed hermaphrodites, i.e., the left side is female and produces an ovary, the right side is male and is capable of producing a testis but this is suppressed in development by hormones emanating from the left ovary. However, in the light of Crew's and Riddle's cases, and from the fact, that the right ovary occasionally persists in birds (Riddle, 1925), it seems pretty clear that Benoit really demonstrated that the sex of birds can be reversed experimentally (Domn, 1924). If we turn from birds to some of the lower vertebrates we find one or two similar if less striking facts. Sex conditions are peculiar in frogs and toads (Hertwig, 1912; Swingle, 1920, 1922, 1925; Witschi, 1914, 1920, 1922). In 1912 Meyns published an account of gonad transplantation in young frogs. He transplanted pieces of the testis of one animal under the skin or into the peritoneal cavity of another. Such grafts undergo considerable degeneration. However, if the transplants were left undisturbed for some months, regenerative processes set in and the germ cells (spermatogonia) developed into oöcyte-like, or egg-like cells, indistinguishable from immature ova. Meyns concluded that the ova-like cells were really ova, and had arisen from the male determined germ cells.

A similar but more striking case is that recently reported by Guyénot and Ponse (1923–1924). They observed that small pieces of the testis of toads when transplanted under the skin of another animal, or in an abnormal position in the same individual, underwent an initial degeneration. Later a regenerative process followed, in which both sperm and egg-like cells developed side by side from sister cells of the same graft. They regard the egg-like cells as immature ova. These early undergo degeneration.

Now, just how are we to regard these cases of apparent sex-reversal in vertebrates? It seems probable that genotypically the cells of the right and left sex glands of embryo fowls are alike, and if we accept the opinion of Meyns and of Guyénot and Ponse with regard to the oöcyte-like cells described by them, then there can be no question but that genotypically identical sex cells, existing side by side are capable of differentiating along male and female lines.

If we assume, as is frequently done by some writers, that maleness and femaleness stand in the relation of simple allelomorphs associated with a single pair of factors, and consequently that the two sexual states are mutually exclusive, we find that hermaphroditism and sex-reversal are utterly inexplicable. However, according to the newer and saner view, the two sexual states appear to result from the interaction of many factor pairs, which severally exist in the allelomorphic condition. The entire complex of genes with male and female tendencies is balanced in a sexually normal individual, and changes in the sexual state (e.g., sex reversal) result from shifting of the balance in a plus or minus direction. It is perfectly obvious that each sex contains the anlagen of the characters of the opposite sex. These are normally not expressed in dioecious organisms because the balance is such that only one of the two sexual states becomes manifest. However, it is conceivable that some radical change in the internal or external environment of the organism, or developing cell, might overthrow the balance of the sex factors established at fertilization and swing the sex in the reverse direction.

Take the case of birds: here we have excellent genetic evidence that under normal circumstances sex in these animals is determined by the chromosome constitution (i.e., the chromosome carries the genetic factors). The complex of genetic factors distributed by the chromosome mechanism is so balanced that one or other of the sexual states is expressed, the other remaining latent. In this group of vertebrates we find also that the sex glands with their hormones are the agents through which the genetic factors work in bringing one or the other sex to full expression. The primary sex-determining agent is the balance of genetic factors as represented by chromosome constitution. These factors give the initial push to sex determination and differentiation, and they are early reinforced by hormones from the gonads which accelerate the process of differentiation. The hormones are the result of other genetic factors and they serve to inhibit definitely the bisexual potentialities of the individual. The evidence from castration and transplantation experiments indicates that the original "genic balance" which served to swing sex definitely one way or the other, is quickly reinforced by accessory sex differentiating agents—the hormones.[2]

Thus we can see how it is possible for a testis-like structure to develop on the right side when the ovary is removed from some birds. It would appear, in such cases, that removal of the inhibiting agent (ovary) tends to overthrow the original "genic balance" established at fertilization so that the latent potentialities of the opposite sex are able to express themselves. So we arrive at the conclusion that sex expression, like the expression of any somatic character, is the result of the interaction of many genes carried in the various chromosomes. It is the expression of a balance struck between the opposing tendencies on the part of the organism. What is really inherited in the matter of sex, as Emerson (1924) phrases it, is "the necessity of reacting in a particular way to a particular internal and external environment." Occasionally violent changes in the internal environment may possibly upset the sex balance and the reaction go in the reverse direction. This does not mean that the sex factors or chromosome constitution themselves change. Far from it, for it is well established experimentally (Morgan, Emerson, etc.) that genetic factors may vary greatly in as far as the effects which they produce are concerned, yet despite the fact the expression of the resulting character is greatly altered, the genetic factor itself remains unchanged. So too with sex reversal, the "genic balance" is upset, the sex reaction reversed, but not a single factor or chromosome is changed thereby, but only the relation in which they stand to one another as parts of this interacting system is altered.

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[2] It is necessary to bear in mind the distinction between sex determination and sex differentiation. In insects the two are much the same, but in vertebrates the zygotic sex-determining factors are limited in their capacities to bring about sex differentiation, and are reinforced by hormones from the sex glands which regulate and control the differentiation processes. By appropriate manipulation of the hormones (extirpation and transplantation) the differentiation process may be reversed in a sexually determined individual.

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THE EFFECT OF BLEEDING UPON THE SERUM CALCIUM OF THYROPARATHYROIDECTOMIZED DOGS[1]

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Investigators of parathyroid tetany have repeatedly called attention to the fact that bleeding the animal during the convulsive attacks and replacement of the blood in part by an indifferent salt solution promptly relieves the symptoms. The present writers have had ample opportunity to confirm this observation during the course of an extensive investigation of parathyroid tetany in dogs. We have observed, however, that bleeding alone is just as effective in relieving tetany as bleeding followed by replacement of an equivalent volume of blood by salt solution. Dogs exhibiting alarming symptoms, such as repeated epileptiform convulsions, can be brought back to an apparently normal condition by withdrawing 100 cc. of blood. The effect of such bleeding upon the tetany symptoms is sometimes very striking; the animal quiets down, the tremors, spasticity and convulsions disappear almost completely, and for several hours afterward the dog appears normal.

Bleeding affords only a temporary relief for sooner or later, generally within six or eight hours, the tetany symptoms return and continued bleeding fails to induce the marked abatement of symptoms which follows the first withdrawal of blood.

The temporary amelioration secured by bleeding a dog in tetany has always been interpreted as the effect of the removal of a circulating poison produced in excessive quantities after removal of the parathyroid glands. It is, in fact, one of the principal supports of the toxin theory of tetania parathyropriva, and an adequate explanation to account for the phenomenon has never been offered by those who deny that parathyroid tetany is primarily the result of a circulating toxin. Voegtlin and MacCallum (1910–11), the founders of the calcium deficiency theory of tetany, state: "This (the fact that bleeding relieves tetany) we have always had to interpret as being the effect of the removal of a circulatory poison produced when the parathyroids are destroyed." Greenwald (1924) makes the

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following statement in his discussion of the toxin theory of tetany: "The usual argument of those who favor the hypothesis that a toxin is the cause of tetany has always been the remission of symptoms and decreased electric excitability of the motor nerves observed after bleeding and injection of salt solution." Berkeley and Beebe (1909) who accept the toxin theory, lay considerable stress upon the remission of tetany symptoms following bleeding as good evidence for their point of view. The following quotation from their paper indicates the importance attached by adherents of the toxin theory to the amelioration of tetany by bleeding. "We have repeatedly confirmed the observation that an animal in severe tetany may be relieved quite promptly by bleeding followed by an intravenous infusion of physiological salt solution. If the tetany is due to a low content of calcium in the blood and tissues such a procedure should make matters worse, because the bleeding may remove one-third of the blood with its low calcium content and the subsequent infusion with salt solution dilutes the remaining blood so that after the operation the tissues are bathed in a fluid having approximately only one-half the calcium content that they previously had, and yet the symptoms have abated. Such a method of treatment, however, is well suited to diminish the concentration of a poison in the circulating blood."

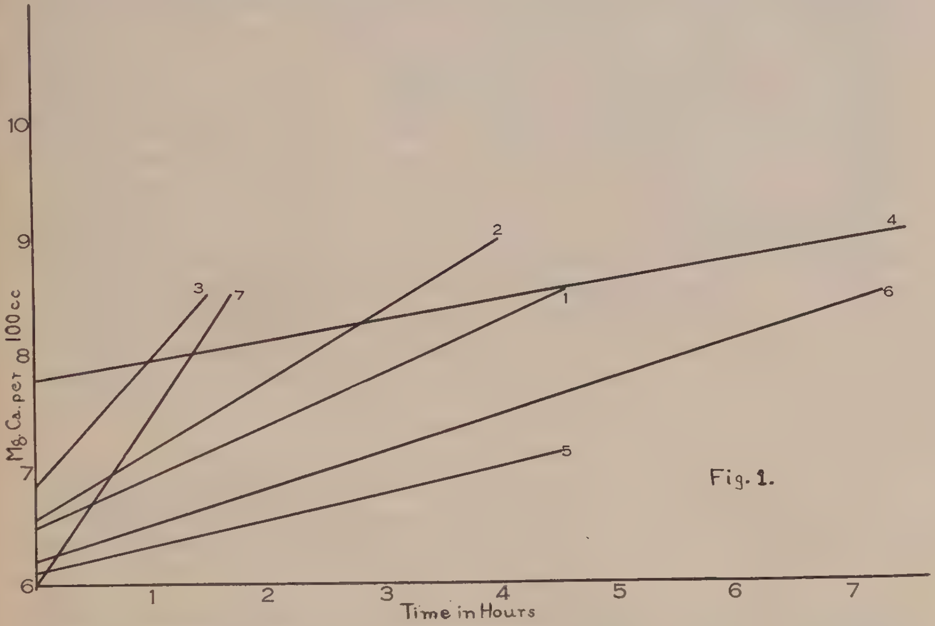

Fig. 1. The serum calcium curve of tetany dogs following bleeding. The numbers represent the experimental animals, the protocols of which are given in the text.

Odd as it may seem, however, the relief of tetany by bleeding, instead of furnishing good evidence that tetany is due to a circulating poison, really affords excellent reasons for denying the existence of any such hypothetical toxin, and gives additional proof that calcium deficiency is the cause of tetany. We have been able to show quite clearly that the withdrawal of large quantities of blood from parathyroidectomized dogs in tetany raises the level of the serum calcium. The abatement of tetany symptoms following bleeding coincides with the rise in the level of the serum calcium, and the subsequent lowering of blood calcium to the level preceding bleeding, which occurs after a few hours, is accompanied by manifest symptoms. Figure 1 shows in graphic form the increase in serum calcium which follows bleeding. No effort was made to measure the calcium level of the serum after the second bleeding except in three cases, consequently our curves do not show the decrease in serum calcium which later occurs. We took the lowering of the calcium level for granted upon the return of tetany, since we have measured the serum calcium of numerous dogs in tetany and have without exception found a great decrease in the calcium content of the serum accompanying manifest symptoms. The tetany level of the serum calcium for dogs seems to be about 7 mgm. per 100 cc. of blood, i.e., tetany becomes manifest when the level of the serum calcium is at or below this figure. There are exceptions to this rule, however, for occasionally dogs with a serum calcium of 7.5 mgm. per 100 cc. exhibit marked tetany (fig. 1, dog 4). The abbreviated protocols of the dogs used in the experiment are given below.

Dog 1. Irish terrier, male, weight 4 kilos. Thyroparathyroidectomized August 6. Serum Ca 10.1 mgm. August 8, violent attack of tetany at 3:30 p.m. Tremors, spasticity, panting and fast pulse. Bled from the heart at 4:15 p.m., 100 cc. of blood taken. Serum Ca 6.5 mgm. At 6 p.m. tetany had abated some. 7:15 p.m., dog quiet and only faint tremors of flank and shoulder muscles observed. 9 p.m., animal perfectly quiet and seemed normal. Bled again at 9 p.m., 35 cc. of blood withdrawn. Serum Ca 8.5 mgm. Animal given 200 cc. of a 5 per cent solution of strontium lactate at 9:30 p.m. and from this time on has been employed for another experiment on tetany prevention.

Dog 2. Fox terrier, male, weight 4 kilos. Thyroparathyroidectomized July 25. Serum Ca, 10.2 mgm. Animal remained normal until July 30 when a violent attack of tetany occurred at 7 a.m. Repeated epileptiform convulsions characterized the attack and artificial respiration was resorted to once in order to save the animal. Bled from the heart at 11 a.m., 30 cc. of blood taken. The animal quieted down and remained free from tetany for twelve hours. Serum Ca not measured. The abatement of symptoms was striking. August 1, animal weak and exhibited marked tremors and spasticity. August 2, violent tetany attack at 10 a.m., 75 cc. of blood withdrawn at 11 a.m. Marked amelioration of the tetany symptoms followed several hours after bleeding. At 3 p.m. the animal was quiet and apparently free from tetany. Bled at 3 p.m., 45 cc. taken. Serum Ca 9 mgm. At 10 p.m. the dog was bled again. Serum Ca 8.2 mgm. No tetany symptoms noted. Bled at 10:40 p.m. Serum Ca 7.8 mgm. August 3, the animal remained free from tetany until 1 p.m. when the tremors returned. The dog died in convulsions at 5 p.m.

Dog 3. English pug, male, weight 3.5 kilos. Thyroparathyroidectomized August 6. Serum Ca 11 mgm. Received 200 cc. of a 5 per cent solution of strontium lactate daily. August 6 to 9, normal. August 10, animal received no strontium. Violent tetany at 12:10 a.m. Epileptiform convulsions. Revived by artificial respiration. Bled at 1:10 a.m., 80 cc. of blood withdrawn. Serum Ca 6.9 mgm. Animal greatly improved after thirty minutes. No signs of tetany. Bled again at 2:40 a.m., 30 cc. taken. Serum Ca 8.5 mgm. The dog remained free from tetany until 8 a.m., when the symptoms returned. This animal was kept alive by strontium administration until August 16, when he died in convulsions. Repeated bleeding on this date failed to raise the Ca level of the serum. (Fig. 2, animal 3.)

Dog 4. Collie, male, weight 10 kilos. Thyroparathyroidectomized July 27. Serum Ca 11.7 mgm. July 28, violent tetany attack. Bled from heart at 10:30 p.m., 80 cc. blood taken. Serum Ca 7.7 mgm. Symptoms abated rapidly, although slight tremors persisted for several hours. 6:30 a.m., July 29, animal showed no tetany symptoms whatever; 35 cc. blood withdrawn. Serum Ca 8.9 mgm. This animal remained normal all day. At 10 p.m. tetany developed. Bled at 11 p.m. Serum Ca 6.4 mgm. July 31, animal in excellent condition. Has but slight tetany symptoms. From this date on the dog was used in another experiment.

Dog 5. Shepherd, male, weight 5 kilos. Thyroparathyroidectomized June 16. Serum Ca 10.2 mgm. June 18, violent tetany attack which continued all day. June 19, animal in bad condition, marked tremors, spasticity and weakness. Bled from heart at 9 a.m., 40 cc. taken. Serum Ca 6.2 mgm. Dog quiet after two hours, and by 11 a.m. tetany symptoms had practically disappeared. Bled again at 1:30 p.m., 35 cc. taken. Serum Ca 7.2 mgm. June 20, animal developed pneumonia symptoms and showed marked tetany tremors. Died the following morning.

Dog 6. Fox terrier, male, weight 3 kilos. Thyroparathyroidectomized August 25. Serum Ca 10.9 mgm. August 26, animal in violent tetany at 8 a.m. Coarse jerkings of limb muscles, panting salivation, spasticity. Bled from heart at 9:20 a.m., 40 cc. blood taken. Serum Ca 6.0 mgm. The tetany attack continued until 1:30 p.m. when the animal became quiet. 4:45 p.m., dog perfectly quiet, no tetany symptoms whatever; 35 cc. of blood taken. Serum Ca 8.4 mgm. Animal remained quiet until the following morning when tetany returned. Bled from the heart at 2:40 p.m. Serum Ca 6.4 mgm; 60 cc. blood taken. Given 200 cc. H_2O, by stomach tube. 5 p.m., animal still in tetany. 11:30 p.m., no change. 8 a.m., August 27, no change. Animal in very bad condition. Given 200 cc. of magnesium lactate. Bleeding experiment discontinued. Animal died 1 p.m., August 27. The rise and fall in the level of the serum Ca is shown in figure 2.

Dog 7. Fox terrier, female, weight 4 kilos. Thyroparathyroidectomized August 24. Serum Ca 11 mgm. August 25, violent attack of tetany at 4:30 p.m. Bled from heart at 7:15 p.m., 110 cc. blood taken. Serum Ca 5.9 mgm. Dog quieted within a few minutes following bleeding. All tetany symptoms had disappeared by 8 p.m., i.e., within forty-five minutes. Bled at 8:40 p.m., 45 cc. blood taken. Serum Ca 8.4 mgm. Animal died at 8:48 p.m. of failing circulation.

The rapidity with which the calcium content of the serum rises after bleeding is sometimes striking. For example, the graph of dog 7 in figure 1 shows that one hour and twenty-five minutes after the withdrawal of 110 cc. of blood, the calcium of the serum rose from 5.9 mgm. to 8.4 mgm. per 100 cc. Animal 3 (fig. 1) showed a somewhat similar curve, although the rise was not so great.

We have not had so much success in raising the calcium of the serum after the second and third withdrawal of blood within twenty-four hours. The animals do not respond nearly so well as they do after the first bleeding. Figure 2 shows the effect of the second bleeding following within a few hours after the first upon the serum calcium of three dogs. The animals were allowed to develop tetany, were then bled and the calcium measured, then bled again after several hours to determine the effect of bleeding upon the calcium level, and finally after a second interval of several hours, bled again. The graphs indicate that the level of the serum calcium is not altered by repeated bleedings after the initial rise. This fact corresponds nicely with our observation that the second and third withdrawals of blood do not greatly affect the tetany symptoms. Apparently, the amount of reserve calcium which the organism is able to mobilize within twenty-four hours under the stimulus of bleeding is limited. It will be noted in figure 2 that the descending limb of the curve of animal 3 is much less abrupt than

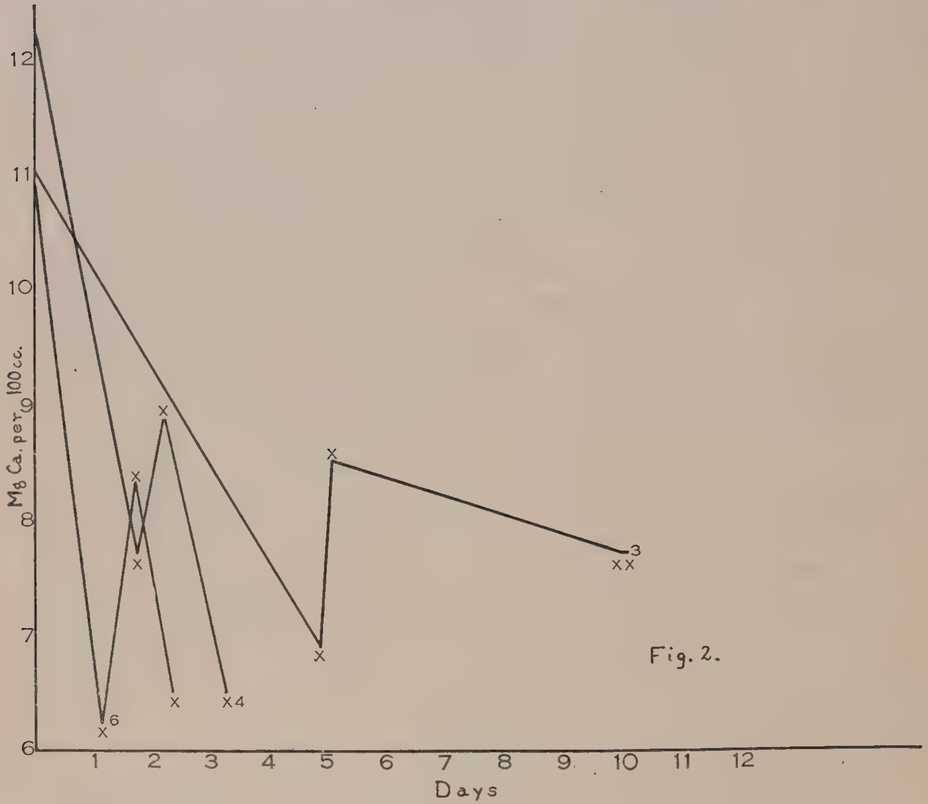

Fig. 2. The calcium curve of three thyroparathyroidectomized animals. The curve begins at the normal Ca level at the time of operation. The crosses indicate withdrawal of blood.

appears in the curve of the other dogs. This is due to the fact that after the rise in the serum calcium, following the first bleeding, had occurred the animal was given 200 cc. of a 5 per cent solution of strontium lactate by mouth, and received this amount daily. We wished to determine whether or not the calcium could be maintained at the new level by strontium.

It is probable that other salt constituents of the blood of dogs in tetany likewise increase temporarily after bleeding, although no attempts were made to determine whether this is the case. The mechanism by which the increase in serum calcium is effected is not clear. It is possible that the sudden withdrawal of considerable quantities of blood from tetany animals enables the organism to tap calcium reserves otherwise not available. However this may be, it seems clear that the remission of tetany symptoms which occurs after bleeding is not necessarily due to diminution in the concentration of a circulating toxin in the blood, but is probably due solely to the increase in utilizable calcium which follows such procedure. Table 1 shows the percentage rise in the serum calcium after bleeding.

TABLE 1

ANIMAL	Ca DURING TETANY	HOURS BETWEEN DETERMINATIONS	Ca AFTER BLEEDING	SYMPTOMS	RISE IN Ca PER CENT
	mgm.		*mgm.*		
1	6.5	4½	8.5	None	24
2	6.5	4	9.0	None	27
3	6.9	1½	8.5	None	16
4	7.7	7½	8.9	None	14
5	6.2	4½	7.2	None	12
6	6.4	7	8.4	None	24
7	5.9	1½	8.4	None	29

SUMMARY

1. Withdrawal of considerable quantities of blood from dogs suffering from tetania parathyropriva promptly relieves the symptoms temporarily, and induces a marked rise in the level of the serum calcium.

2. The abatement of symptoms coincides with the rise in serum calcium. Within ten or twelve hours after bleeding the calcium content of the blood again decreases and tetany becomes manifest.

3. The remission of tetany symptoms which follows bleeding cannot be considered good evidence that such procedure diminishes the concentration of a toxic substance in the circulating blood.

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THE PREVENTION AND CURE OF TETANY BY ORAL ADMINISTRATION OF STRONTIUM[1]

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Since the publication of MacCallum and Voegtlin's (1909) pioneer work on the relation of the parathyroid glands to calcium metabolism, other investigations have appeared dealing with the effects of various agents, both physical and chemical, upon the tetany syndrome. Several methods of medication and treatment besides calcium administration have been found to temporarily relieve, or even permanently cure, tetany in dogs. The following procedures have all been found efficacious: intravenous injections of $\frac{M}{1}$ NaCl solution (Joseph and Meltzer, 1910–11), bleeding, intravenous injections of strontium and magnesium (Berkeley and Beebe, 1909), intravenous injections of Ringer's solution (Luckhardt and Rosenbloom, 1922), addition of large quantities of lactose to the diet (Dragstedt and Peacock, 1923), and ultra-violet radiation (Swingle and Rhinhold, 1925).

There is little agreement as to the mechanism involved in the relief, prevention or cure of tetany, as the case may be, by the various agents and procedures mentioned. Some believe that the prompt relief of tetany which follows bleeding or the continuous intravenous injection of Ringer's solution, with consequent diuresis, is due to ridding of the body of a circulating toxin. Others think that the prevention of symptoms by adding lactose to the diet, is due to changes induced in the metabolism of the intestinal flora whereby certain toxic substances (assumed to be the cause of tetany) due to bacterial decomposition of proteins are not produced. The amelioration of tetany symptoms following strontium and magnesium injections is generally stated to be due to the sedative influence of these salts upon the nervous system.

It is our belief, however, that most, if not all, of the agents named are effective in the treatment of parathyroid tetany only insofar as they affect the retention and utilization of calcium by the organism, and per se are capable of exerting little influence. Some previous work (Swingle and Rhinhold, 1925; Swingle and Wenner, 1925) had shown that the ameliorative action of ultra-violet radiation upon tetany is probably due to its action on calcium metabolism, and also that the prompt relief of tetany symptoms which follows bleeding is due solely to the rise in the level of the serum calcium which occurs after such procedure. In the present communication, data are presented indicating that the preventive and curative action of strontium upon tetany is due to the action of this salt in promoting calcium retention after parathyroidectomy rather than to any inherent curative properties possessed by the salt itself.

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Berkeley and Beebe (1909) were the first to call attention to the fact that strontium chloride, when administered intravenously to a dog in tetany, promptly relieves the symptoms. The effect of strontium salts was tried upon ten dogs in tetany, and the conclusion reached that it is scarcely possible to decide whether calcium salts give any better results. The effects of the calcium appear a little earlier than those of strontium, but they are not more complete or lasting. None of Berkeley or Beebe's animals receiving the strontium lived more than a few days. The symptoms could be kept under control, but the dogs invariably died of nutritive disturbances.

In the following year, Voegtlin and MacCallum (1910–11) published the results of their investigation on the influence of various salts upon tetany. They also used strontium chloride intravenously and stated that on the whole, the effects of this salt are similar to those produced by calcium, although the lowering of the excitability of the motor nerves to electrical stimulation is not quite so marked. Despite the abatement of tetany symptoms following injection of strontium, Voegtlin and MacCallum were unable to preserve the life of the dogs for more than a few days, the animals dying of malnutrition and exhaustion. However, one animal survived fifteen days.

Considerable experience with parathyroidectomized mammals (Swingle and Nicholas, 1924–25), and especially work on the effect of repeated intravenous and subcutaneous injections of calcium in tetany dogs, led the present writers to believe that such treatment has probably killed almost as many dogs as the tetany itself. We considered it probable that oral administration would be as effective and much simpler. It will be recalled that Luckhardt and Goldberg (1923) were able to prolong indefinitely the lives of their animals by calcium feeding. Our experiments were under way when a short abstract by Dragstedt (1925) appeared in the Proceedings of the American Physiological Society, which dealt with similar experiments.

Dragstedt used nine dogs, and following parathyroid removal the animals were fed bread and water ad libitum. Fifteen grams of strontium lactate dissolved in water or incorporated in corn meal mush were given by stomach tube. Seven animals died of tetany at the end of 4, 7, 8, 11, 17, 18 and 23 days. Two recovered and were alive eight months after the operation. The animals occasionally developed tetany which could be completely controlled by intravenous injection of from 800 to 1200 cc. of modified Ringer's solution in which strontium chloride replaced the calcium of the usual formula. Dragstedt states that strontium is not so effective as calcium in preventing tetany.

We are not aware of any other investigations besides the three mentioned (one reported in an abstract of about one hundred words) and our own, which deal with the relation of strontium to tetany. The essential problem with which we are here concerned is, how does strontium act in preventing or relieving tetany?

Twenty-seven dogs were used in the investigation. Careful attention was given to the diet, since it is well established that certain diets tend to induce tetany, e.g., meat, whereas others such as white bread and milk, or milk and lactose prevent—or at any rate—greatly retard the onset of tetany in operated dogs. The diet employed was a modification of that recommended by Cowgill (1923) for metabolism experiments, and contains casein, sucrose, lard, butter and a salt mixture from which we omitted the calcium (Swingle and Rhinhold, 1925). Sixteen grams of this diet per kilo of body weight per day are sufficient to maintain a dog in excellent physical condition. We have tested the diet on parathyroidectomized dogs and find that it has no preventive effect on tetany. Hence the diet must be ruled out as a factor in any way influencing the results of our experiments.

Strontium chloride was first employed but soon abandoned because of its irritating action on the gastro-intestinal tract. Dogs receiving the chloride refused food and invariably died of nutritive disturbances, although the tetany symptoms could be controlled. Strontium lactate was found to be as effective as the chloride but not so irritating. Immediately following the operation, when the effects of the anesthetic had passed, the animals were given 200 cc. of a 5 per cent solution of strontium lactate by stomach tube. This amount was given daily.[2] Table 1 shows the effects of strontium in preventing tetany.

It will be noted that some animals lived for comparatively long periods and probably could have been kept indefinitely had it been desirable. We were forced to get rid of all dogs surviving more than 72 days because of inadequate space for keeping large numbers of dogs in the laboratory. For instance, animals 2, 3, 8, 9, 11 and 12 were apparently normal dogs at the end of the 72 days and in excellent physical condition. These

[2] It was later found that 100 cc. given three times daily was more effective than a single massive dose.

animals received 200 cc. of strontium lactate daily for about 40 days, except animal 12 (see protocol). At the end of this time the strontium was discontinued but the animals were kept on the diet. On the 50th day a meat diet was substituted. None of the dogs showed tetany symptoms following discontinuance of the strontium and replacement of the special diet by meat.

TABLE 1

The effect of strontium in the treatment of tetany

ANIMAL	SEX	OPERATED	TETANY	RESULT	DURATION EXPERIMENT days	REMARKS
1	♂	March 19	March 22	Died	9	Animal received strontium chloride
2	♂	March 30	April 1	Recovered	72	
3	♂	April 7	April 9	Recovered	72	
4	♀	April 21	April 26	Died	23	Animal pregnant
5	♂	April 1	April 8	Died	14	Died of pneumonia
6	♀	April 8	April 9	Died	15	Dog pregnant
7	♀	May 5	May 16	Died	15	Advanced pregnancy
8	♂	May 19	May 23	Recovered	72	
9	♂	May 22	May 25	Recovered	72	
10	♀	April 21	May 11	Died	21	Advanced pregnancy
11	♂	May 26	May 30	Recovered	72	
12	♂	August 6	August 8	Recovered	72	
13	♀	May 8	May 14	Died	11	Pregnant
14	♀	June 11	June 15	Died	11	Pregnant
15	♂	June 12	June 14	Died	4	
16	♂	June 12	June 18	Died	8	
17	♂	June 14	June 17	Died	7	
18	♂	June 13	June 15	Died	27	
19	♂	June 16	June 20	Died	18	
20	♂	June 17	June 19	Died	2	
22	♂	July 14	July 20	Died	10	
23	♂	July 24	August 2	Died	9	
24	♂	July 24	July 30	Died	14	
35	♂	August 6	August 14	Died	8	
36	♂	August 14	August 19	Recovered	72	
37	♂	August 15	August 23	Died	8	
38	♂	August 15	August 27	Died	12	

There can be no question of parathyroid tissue being left behind at operation because all of the animals developed tetany symptoms, three were in very bad shape for several days and little hope was held for their recovery (see protocols). The animals could be thrown into tetany at will up until the 30 or 35th day by merely discontinuing the strontium. After this interval had elapsed since operation the dogs seemed to recover completely. The following abbreviated protocols of five dogs will give some idea of the experiments.

Protocol, Dog 2. Adult male, weight 8 kilos. Thyroparathyroidectomy March 30. Fed some meat along with regular diet. Given 200 cc. of 5 per cent solution of strontium lactate daily.

April 1, tetany.
April 2–13, mild tetany; animal losing weight rapidly.
April 14, dog has pneumonia symptoms and marked tetany.
April 15–20, animal very weak and emaciated. Fed by stomach tube. Tetany abating.
April 21–25, dog much better, eats and is recovering strength. Occasionally has mild attacks of tetany.
April 25–27, tetany symptoms have entirely disappeared. Animal appears normal.
April 28–May 18, dog perfectly normal; has gained much weight. Strontium discontinued May 18.
May 19–May 27, dog normal. Serum Ca, May 22, 10.2 mgm.
May 31, casein diet discontinued and heavy meat diet substituted. Serum Ca, May 20, 10.2 mgm.
June 1–15, animal normal. Killed June 16. Autopsy revealed no accessory parathyroids. Serum Ca, June 15, 10.4 mgm.

Protocol, Dog 3. Adult male, weight 4 kilos. Parathyroidectomized April 7. Received 200 cc. strontium lactate daily.

April 9–10, marked tetany.
April 11, tetany abating.
April 12, animal normal except for weakness.
April 13, fed meat. Within two hours had a violent attack of tetany. Given 400 cc. strontium. No tetany after three hours.
April 14–21, animal normal.
April 21–29, normal, struggled violently upon passage of stomach tube on April 29 and developed violent attack of tetany a few hours later.
April 30, tetany. Animal so spastic it is unable to walk.
May 1–3, tetany continues but it is not violent.
May 4, tetany abating.
May 5, animal shows no tetany symptoms; seems much stronger.
May 6–28, dog normal. Strontium discontinued May 28. Serum Ca, May 22, 10.5 mgm.
May 29–June 11, animal normal; gaining weight. Casein diet replaced by meat. Serum Ca, June 1, 10.6 mgm. June 8, 11 mgm.
June 20, animal killed. Accessory glands not found at autopsy.

Protocol, Dog 8. Adult male, weight 6 kilos. Operated May 19. Received 200 cc. of 5 per cent solution strontium lactate daily.

May 20–22, dog normal.
May 23, tetany.
May 24, mild tetany; symptoms abating.
May 25–July 6, animal normal. Strontium discontinued; meat diet substituted for casein.
July 6, serum Ca, June 10, 10.9 mgm.
July 7–August 1, dog normal. Killed August 21. Serum Ca, June 24, 10.1 mgm. August 1, 10.7 mgm.

Protocol, Dog 9. Adult male, weight 6 kilos. Thyroparathyroidectomized May 22. Given 200 cc. strontium daily.
May 25, animal has tetany.
May 26–28, dog in bad shape. Losing weight rapidly.
May 29–June 3, tetany abating. Dog seems better.
June 4–6, no tetany.
June 7–17, animal normal. On the 17th the animal engaged in a fight with another dog and was severely bitten. Mild tetany supervened some hours later. June 11, serum Ca, 10.4 mgm.
June 18, mild tetany. Animal spastic.
June 19, dog normal.
June 20–August 1, normal; strontium discontinued July 6, and meat substituted for the casein diet. Serum Ca, June 24, 10.6 mgm., August 1, serum Ca, 10.8 mgm.

TABLE 2

Effect of strontium in delaying the onset of tetany

ANIMALS NOT RECEIVING STRONTIUM*						ANIMALS RECEIVING STRONTIUM					
Animal	Operated	Ca normal	Tetany	Ca	Interval Operation-Tetany	Animal	Operated	Ca normal	Tetany	Ca	Interval Operation-Tetany
		mgm.		*mgm.*				*mgm.*		*mgm.*	
25	June 10	11.2	June 12	5.8	2	22	July 14	11.3	July 10	6.7	6
26	June 12	10.8	June 14	6.1	2	23	July 24	10.5	August 2	6.1	9
27	June 12	11.1	June 14	6.1	2	24	July 24	10.5	July 30	7.4	6
28	June 15	10.7	June 18	5.7	3	35	August 6	11.1	August 14	6.4	8
29	June 17	10.9	June 18	6.4	1	36	August 14	11.0	August 19	6.9	8
30	July 27	11.3	July 30	6.3	3	37	August 15	10.2	August 23	6.5	8
31	July 27	12.2	July 29	6.7	2	38	August 15	10.8	August 27	5.9	12
32	August 5	10.1	August 8	6.5	3	5	April 1		April 10		9
33	August 10	10.2	August 11	6.7	2	10	April 21	11.0	May 11	6.9	20
34	August 16	10.6	August 13	6.4	1	7	May 5	11.0	May 17	6.7	12

* The animals of the series not receiving strontium were used for another experiment run simultaneously with that on strontium and serve as controls for the latter.

Protocol, Dog 11. Adult male, weight 7 kilos. Operated May 26. Serum Ca, 10.8 mgm. Strontium given daily.
May 30, tetany.
May 31–June 3, dog in bad condition; very weak.
June 4, animal much better. Tetany abating.
June 5–July 6, dog normal. Strontium discontinued on July 6, and the casein diet replaced by meat. June 10, serum Ca, 10.3 mgm., June 24, Serum Ca, 10.6 mgm.
August 1, 10 mgm.

The question arises—how does strontium prevent tetany? Our data indicate that the mechanism by which strontium prevents tetany is chiefly in preventing calcium excretion. Salvesen (1923) has conclusively shown that the loss of calcium from the organism following parathyroid removal is due to rapid elimination of this salt through the gut wall. In view of his results, our data seem to indicate that strontium acts upon the intestinal tract in such a manner as to decrease its permeability to calcium so that the latter is not permitted to escape. As a consequence of the decreased permeability of the gut to calcium excretion, the amount of this salt in the blood and tissues is not appreciably diminished following parathyroid removal. We have repeatedly observed operated dogs kept free from tetany by strontium, and with normal blood calcium, develop violent tetany accompanied by greatly diminished amounts of calcium in the serum, following cessation of the strontium treatment. Once the animals develop tetany, however, it is very difficult to cure them by strontium administration. The serum calcium remains low and is only slightly increased by strontium feeding. Table 2 shows the striking effect of

TABLE 3

Effect of strontium administration on the level of the serum Ca

ANIMAL	Ca AT OPERATION	DATE	Ca DETERMINATIONS			REMARKS
9	Not determined	May 22	June 11 10.4 mgm.	June 24 10.6 mgm.	August 1 10.8 mgm.	Animal had tetany from May 25–June 3. Recovered completely
8	Not determined	May 19	June 10 10.9 mgm.	June 24 10.7 mgm.	August 1 10.7 mgm.	Animal had tetany from May 23–29. Recovered completely
3	Not determined	April 7	May 22 10.5 mgm.	June 1 10.6 mgm.	June 8 11.0 mgm.	Tetany from April 9–16. Recovered completely
2	Not determined	March 30	May 22 10.2 mgm.	May 30 10.2 mgm.	June 15 10.4 mgm.	Tetany from April 1–23. Recovered completely
11	10.8 mgm.	May 26	June 10 10.8 mgm.	June 24 10.6 mgm.	August 1 10.0 mgm.	Tetany from May 30–June 3. Recovered
12	10.1 mgm.	August 6	6.5 (8.5)*	8.5	11.0	Tetany August 8. Bled. Complete recovery

* Animal developed violent tetany. Bleeding raised the serum Ca to 8.5 mgm. within a few hours; no further tetany after strontium administered.

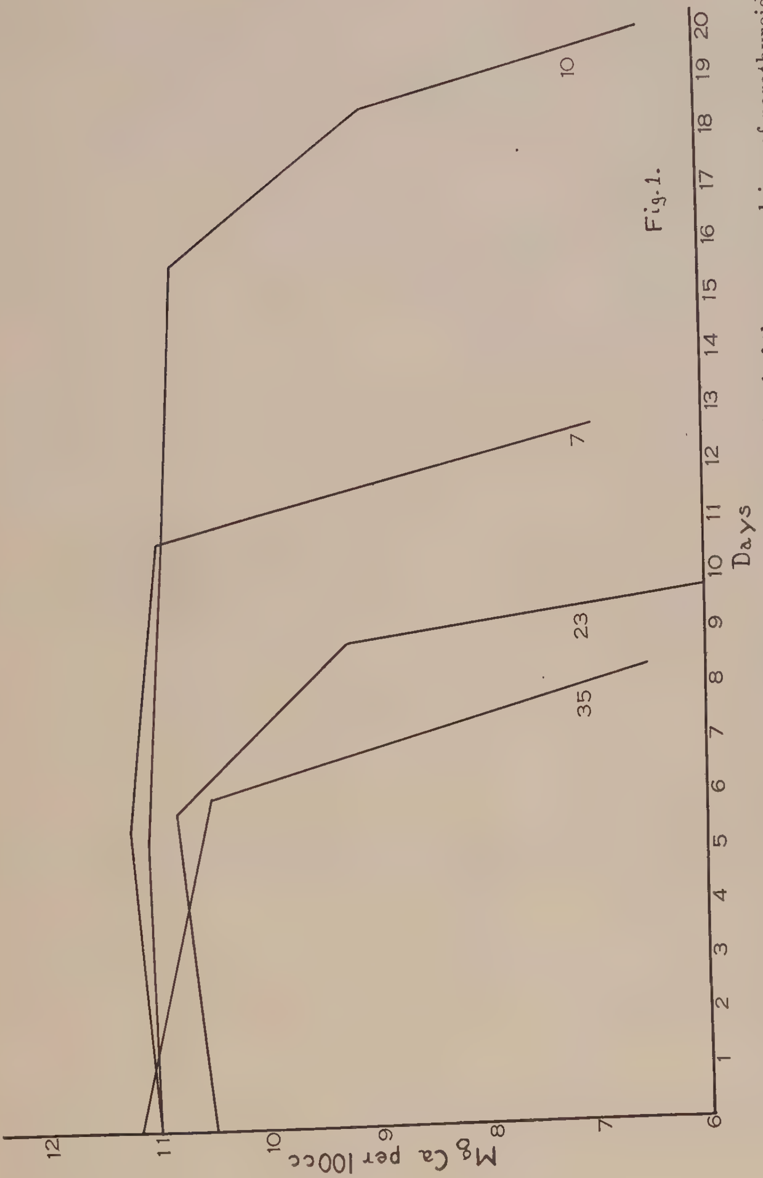

Fig.1.

Fig. 1. The effect of strontium upon the maintenance of the normal level of the serum calcium of parathyroidectomized dogs. Tetany symptoms became manifest when the Ca fell to 7 mgm. per 100 cc.

strontium in delaying the onset of tetany symptoms in operated animals, and table 3 shows the effect of the salt in maintaining the normal level of the serum calcium.

Table 2 shows in unmistakable manner that operated dogs receiving strontium may go for ten days or two weeks without developing tetany symptoms, whereas untreated dogs invariably show violent tetany within three or four days or even within twelve hours after parathyroid removal. Operated animals free from symptoms also show a normal blood calcium level (table 3 and figure 1). When tetany becomes manifest, however, the serum calcium is always low. This fact indicates that strontium somehow prevents loss of calcium from the blood and tissues and assists in maintaining the normal level of this salt in the blood. Figure 1 shows the effect of strontium administration upon the level of the serum calcium of parathyroidectomized dogs.

We have repeatedly observed that dogs which developed tetany can be brought back to normal within a few days by continuous treatment with strontium and feeding one pint of milk per day. If the milk alone is given and no strontium, the animal soon dies, and if the strontium alone is given, say with meat or the diet employed by us, it is very difficult to save the animal or to raise the level of the serum calcium. These observations made it evident that something in the milk aided the animals to recover when given strontium. Salvesen (1923) has clearly shown that this something in milk is calcium, for if large quantities of milk are fed to parathyroidectomized dogs the animals can be kept alive for long periods. In our animals which recovered completely from tetany on a milk (limited amount) and strontium diet, the strontium evidently decreased the permeability of the gut to Ca excretion so that whatever entered the body as food was retained and utilized.

We observed that coincident with the abatement of tetany symptoms and a return to normal of our milk and strontium-fed dogs, the calcium level of the serum rose to normal, i.e., from about 6.5 mgm. per 100 cc. to about 10.5 mgm.

One of the most crucial bits of evidence that oral administration of strontium prevents tetany by inhibiting calcium excretion is shown by the following experiment. We had previously noted (Swingle and Wenner, 1925) that bleeding an animal in tetany promptly relieves the symptoms which completely disappear for several hours. The abatement of the symptoms coincides with a marked rise in the level of the serum calcium which follows withdrawal of blood. This rise is temporary and the serum calcium soon falls again to the tetany level when manifest symptoms return. However, we have several dogs in which we were able to maintain the high calcium level of the blood induced by bleeding, by administering strontium after the peak of the rise had been attained. By continuing

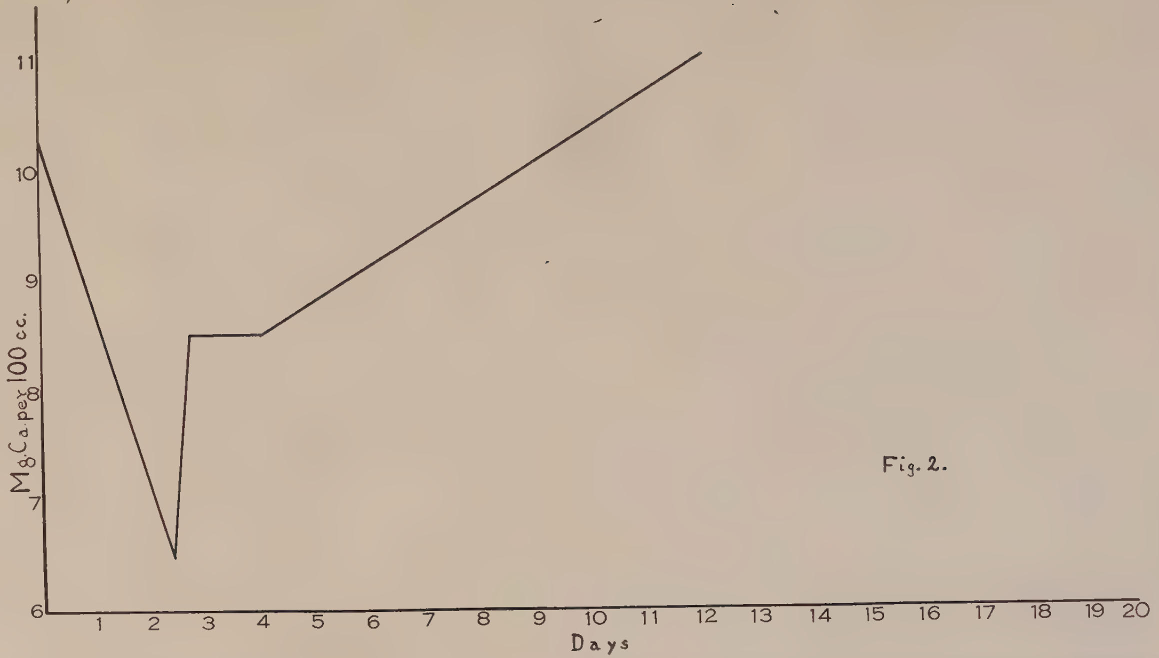

Fig. 2. The effect of bleeding upon the level of the serum calcium of a dog in tetany, and the maintenance of the new level by strontium.

the strontium treatment the serum calcium did not fall but was kept at the new level and from which it slowly rose to normal. Figure 2 shows the effect of bleeding upon the level of the serum calcium of a tetany dog, and the maintenance of the new level by strontium administration. The protocol of the animal is given below.

The cause of the cure effected in this case is not far to seek. The strontium simply decreased the permeability of the gut so that the new high level of the calcium in the circulatory fluids of the body was not lost by excretion. If the strontium is discontinued the serum calcium sinks to the tetany level and the animal dies.

We believe that any substance which serves to decrease the permeability of the gut to calcium excretion will prevent or cure tetania parathyropriva. There is probably a considerable number of such substances. Recent observations we have made, indicate that magnesium lactate acts in the manner indicated, and it seems likely that both lactose and galactose, and continuous intravenous injections of Ringer's solution act similarly. The marked effect of a meat diet in hastening the onset of tetany in operated animals has been noted by many observers and variously explained. We believe that the meat diet leads to great increase in intestinal putrefaction (shown to be the case by Dragstedt, 1923), and it is probable that several of the end products of bacterial decomposition of protein promote the onset of tetany by increasing the permeability of the gut to calcium excretion. We have observed that dogs kept free from tetany by strontium, very quickly develop violent symptoms if constipated. This is also true for dogs on a lactose diet, and even those receiving daily amounts of calcium. Constipation, like a meat diet, predisposes a parathyroidectomized dog to tetany. This fact has been clearly recognized by other workers (Luckhardt and Rosenbloom, 1922; Dragstedt and Peacock, 1923).

Protocol, Dog 12. Adult male, weight 4 kilos. Operated August 6; no strontium given. August 8, violent attack of tetany at 3:30 p.m. Bled from the heart at 4:15 p.m., 100 cc. of blood taken. Serum Ca 6.5 mgm. At 6 p.m. tetany had abated some. At 7:15 p.m. dog quiet except for five tremors of flank and shoulder muscles. 9 p.m., dog quiet; no tetany symptoms. Bled again at 9 p.m.; 35 cc. blood withdrawn. Serum Ca 8.5 mgm. Animal given 200 cc. of a 5 per cent solution strontium lactate at 9:30 p.m. August 9–October 16, dog normal. Strontium discontinued August 28. August 28, meat substituted for the casein diet. Serum Ca August 10, 8.5 mgm.; August 18, Ca 11 mgm.; August 27, Ca 11 mgm.

Besides its preventive action on tetany by decreasing the permeability of the gut to calcium excretion, strontium exerts a direct ameliorative action upon tetany. Violent symptoms can be promptly controlled by either intravenous or oral administration of strontium salts. The strontium apparently numbs the motor nerves, so that a much greater stimulus is required to induce the neuromuscular symptoms. Like calcium, it has a sedative effect upon the nervous system, and its action in relieving the alarming symptoms is of this nature, rather than by any power on the part of the organism to substitute this salt for calcium, although the latter possibility cannot be ruled out.

Voegtlin and MacCallum (1909) demonstrated that strontium acts like calcium in lowering the electrical excitability of the motor nerves, but that the effects were not quite so marked. However, one cannot cure tetany by drugging an animal, for despite the absence of manifest symptoms, the dog still suffers from loss of its parathyroids. This is shown by the continual loss of weight, depression and low blood calcium. Only by careful nursing and forced feeding is it possible to bring the animal back to normal, with a normal serum calcium level.

Hence it appears probable that strontium exerts a twofold action in preventing and relieving tetany: 1, it prevents or delays the onset of tetany symptoms by decreasing the permeability of the gut to calcium excretion so that the level of the calcium in blood and tissues remains above the tetany level; and 2, it relieves the symptoms once they appear by its sedative action on the nervous system, so that a much greater stimulus is required to induce the neuro-muscular symptoms.

It was previously mentioned that dogs kept free from tetany for several weeks could be taken off the strontium regime without developing symptoms, and after the 50th day could take the ordinary diet of meat and bread. Previous to this time such a diet would induce fatal tetany. The animals are thus able to readjust themselves to the loss of their parathyroid glands after four or five week's treatment, and are in all respects normal dogs. This is a curious fact and one not easily explained. Other investigators who have worked on tetany prevention have made similar observations but with the exception of Dragstedt and Peacock (1923) have offered no comments. Thus Luckhardt and Rosenbloom (1922) observed that completely parathyroidectomized dogs can be kept alive indefinitely by daily intravenous injections of large quantities of Ringer's solution. They remark that after four or five weeks of such treatment the injections can be discontinued and the animals remain normal even though fed meat daily.

Dragstedt and Peacock (1923) state that dogs kept free from tetany for five or six weeks by a lactose diet can be given a meat diet at the end of this time and remain normal.

There are two possibilities to account for such readjustment: 1. It is possible that small accessory parathyroid glands, or bits of tissue, may be present in dogs which are unable immediately to take over the function of the main mass of parathyroid tissue removed at operation, but which are able to function adequately after the animal has been tided over the first few critical weeks. 2. It is possible that some other organ has taken over the function of the missing glands and is acting vicariously for them.

Dragstedt particularly favors this view and suggests that the liver may be involved.

The evidence is quite clear that parathyroidectomized dogs can be permanently cured if kept alive for four to six weeks by various experimental procedures. The results of continuous intravenous injections of Ringer's solution, special diets containing lactose and galactose and strontium all give identical results. But the mechanism of such readjustment to parathyroid loss is not clear. It is our opinion that the majority of dogs, in common with several other mammals, e.g., man and the cat, possess minute particles of parathyroid tissue scattered throughout the throat region and especially in and about the upper portion of the thoracic thymus. We have not been successful in finding any such accessory tissue at autopsy, but anyone at all familiar with accessory parathryoids will realize the improbability of identifying such tissue by macroscopic examination. Very small particles of parathyroid tissue are sufficient to keep a dog alive and free from tetany. One of the writers (W. F.W.) in a series of autoplastic transplantation experiments (unpublished) has demonstrated that one-half of a single parathyroid gland is ample to maintain a dog in a normal healthy condition. A piece of tissue of the size mentioned is entirely too small to be readily detected at necropsy—no matter how carefully conducted.

We have sectioned large portions of the thymus of dogs surviving parathyroidectomy after prolonged treatment, but have not found accessory glands. Nevertheless, despite the negative findings it is our belief that permanent cures of parathyroidectomized dogs after a return to a meat diet, and with a serum calcium level of 10.5, can only mean the presence of functional accessory parathyroid tissue somewhere in the body.

In the course of an extensive investigation of the parathyroid apparatus of cats (Nicholas and Swingle, 1924–25) the senior writer (W. W. S.) had opportunity to observe the efficient functioning of accessory parathyroids. These structures are quite common in cats. In fact, 35 per cent of these animals have them. They are capable of functioning for the entire parathyroid apparatus when the latter is absent. Marine (1913) states that his data indicate that 5 to 6 per cent of dogs have accessory parathyroid glands. During the past two years we have operated on more than seventy dogs and have noted but two animals that failed to develop tetany following complete removal of the thyroparathyroid apparatus.

The onset of tetany in dogs is sudden and violent, and the animal quickly succumbs before the accessory tissue present has time to take over the function of the removed glands. However, it seems probable that by tiding the animal over the first few critical weeks any such accessory tissue has time to hypertrophy and function adequately. At least three weeks are required before accessory parathryoid tissue can function sufficiently to keep the dog normal. We have observed several animals receiving strontium, which remained free from symptoms for eighteen or twenty days after operation, suddenly develop violent tetany. The protocol of dog 3 is of interest in this connection.

SUMMARY

1. Tetany in dogs can be prevented from developing, can be relieved or even cured by continuous oral administration of strontium lactate.
2. The strontium acts in two ways: 1, by decreasing the permeability of the gut to calcium excretion so that the normal level of this salt is maintained in the blood and tissues; and 2, by a sedative effect upon the nervous system whereby the excitability of the motor nerves is greatly diminished.
3. Thyroparathyroidectomized dogs kept free from tetany by strontium for about 40 days readjust themselves to the loss of their parathyroids and permanently recover.
4. Several suggestions as to the mechanism involved in such readjustment are offered in the text.

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EXPERIMENTS ON THE DEVELOPMENT OF THE HEART OF AMBLYSTOMA PUNCTATUM[1]

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THIRTY-NINE FIGURES

CONTENTS

Introduction 321
Material, methods, and normal development 326
Experimental 333
  A. Extirpation of heart rudiment 333
  B. Transplantation of rudiments 339
  C. Effect of superimposing heart rudiments 345
  D. Splitting rudiment into right and left halves 348
  E. Extirpation of parts of rudiment 351
  F. Transplantation of parts 358
Discussion 361
Summary 367
Literature cited 369

INTRODUCTION

Experimental work bearing directly on the development of the heart has been limited almost entirely to studies of very recent date. Closely associated problems have, however, been extensively studied. The movement of the heart has been shown to be autonomic by study of the heart in tissue cultures, Burrows ('11) and Braus ('12), and, by study of the heart in embryos from which the nervous system had been removed, Hooker ('10 and '11). Similar results have been obtained by others and results of the present study also show that the initiation of the heart beat is independent of the nervous system and that the nerve supply of the heart functions merely as a regulatory agent.

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[1] Read before the American Society of Zoölogists, December 30, 1924.

The development of the embryo in the absence of a functional heart has been shown to occur in the amphibian by Knower ('07) and Clark ('18), in the chick by Chapman ('18), and in the teleost by Loeb ('97) and Stockard ('07). In the present study it has also been found that Amblystoma embryos lacking a functional heart develop in a fairly normal manner during the first few days following the operation and continue to live for three or four weeks. The larger part of the present study deals, however, more with the progressive differentiation of the heart from its embryonic rudiment and with the equipotentiality of the rudiment.

That the rudiment of the heart is of a plastic nature rather than a fixed mosaic has been indicated by the types of malformations described in pathological studies and also by duplicities that have been described. The most remarkable of multiple formations is that described by Verocay ('05) of a hen having seven hearts of equal size in place of the normal one. That the heart rudiment is in its early stages of development of an exceedingly plastic nature has also been shown by experimental work. Born ('97) described various degrees of fusion of the hearts when embryos of Rana esculenta were joined along the ventral surface. Fusion of two rudiments in this way would indicate that the rudiment of the heart is not of a mosaic nature. A similar conclusion would be drawn from the work of Gräper ('07), who, by preventing the union of the halves of the rudiment in the chick embryo, that is, by hindering the joining of the omphalomesenteric veins, caused the development of two hearts of normal size in one embryo. The work of Levy ('06), on the other hand, would indicate that the rudiment is of a mosaic nature. Levy divided embryos of Triton taeniatus into anterior and posterior parts and has described cases in which the division was through the heart rudiment. In one case the bulbus of the heart was entirely lacking and in other cases parts were found to be relatively poorly developed, indicating that the rudiment was not equipotential.

The first extensive experimental work on the development of the amphibian heart was done by Ekman ('21) on embryos of Bombinator. He has described complete development after removal of parts of the rudiment at an early stage: development of two hearts, circulation being absent, after splitting of the rudiment in the midline, and development of the heart within a ball of ectoderm when explantations of ectoderm and mesoderm were made. In one case a transplantation was made in which the heart rudiment was rotated 180° so as to place the anterior part posteriorly, and the development indicated that the postero-anterior axis had not been determined within the rudiment at the time of the operation. From the results of various types of operations, Ekman concluded that the heart of Bombinator was a self-differentiating, harmonic equipotential system.

Stöhr ('24 b) performed a large number of explantation experiments with Bombinator and, like Ekman, found that the heart would develop in an explanted ball of ectoderm. Stöhr, however, emphasized the importance of 'Entomesodermzellen,' which were always included in the explant, and concluded, accordingly, that the heart was not self-differentiating to as great extent as Ekman's work had indicated. The form of the heart developing in explantation experiments was found to be variable, and Stöhr concluded that there was within the rudiment itself a tendency for curving, as had been stated by Spemann ('06) and by Pressler ('11), but that the particular type of curvature was the result of outside factors. It was found that two organs could be made to develop from one rudiment by splitting the rudiment in the midline, but in the explantation experiments, when a part of the rudiment was explanted, only a fragmentary heart developed, and Stöhr accordingly concluded that the results did not fulfill the requirements of an equipotential system. Stöhr ('24 c and '24 d) has also described in heterotopic transplantation experiments unequal differentiation of parts as additional evidence not agreeing with the view that the heart is an equipotential system. Ekman ('24 b), on the other hand, as evidence for calling the heart an equipotential system, has described as many as three hearts of all parts developing from one rudiment.

As evidence for self-differentiation, Ekman ('24 a) has described the development of a pulsating heart in Ringer's solution in the absence of neighboring tissues, indicating the relative unimportance of the latter. The hearts developing in Ringer's solution showed, however, very little differentiation, and a question might be raised with respect to the degree of development and differentiation which should take place in order for an organ to be called self-differentiating. On the question of the relative importance of neighboring tissues and on the question of the amount of self-differentiation which should be expected in a self-differentiating system, Ekman and Stöhr seem to differ. On the question of the development of the form of the heart, they agree that the factors are: self-differentiation ability, influence of the blood stream, and correlative influence of neighboring tissue. Ekman has especially studied and emphasized the first of the factors mentioned, and Stöhr, the latter two. In heterotopic transplantation experiments Stöhr ('24 d) has considered the importance of the blood stream not only as a factor for the form development of the heart, but also as a factor for growth and development of the organ itself. When one or both ends of the heart were joined with vessels of the host, more development and differentiation took place than when union with vessels of the host did not accur.

Concerning the effect of rotation, Stöhr ('24 d) stated that when the rudiment was rotated 180°, placing anterior part posteriorly, the heart developed in a rotated manner. This result was apparently not in agreement with Ekman ('21) that at the closed medullary-fold stage of development the rudiment developed according to influence of the environment and rotation of the rudiment did not produce rotation of the developed heart. Stöhr, in a later paper (Schübel and Stöhr, '24), has briefly described development in Bombinator after rotation of 180°, stating that rotation of the rudiment is followed by normal development when the operation is made in the open medullary-fold stage, but that a similar operation at the tail-bud stage is followed by development of a reversed heart which retains its original direction of movement.

From the foregoing review of the literature, it is evident that there are differences in opinion concerning the ability of the heart for self-differentiation and concerning the question of equipotentiality. An interesting question also arises concerning the effect of rotation and its relation to equipotentiality; in other words, is the heart equipotential at the same stage in development at which rotation of the rudiment produces rotation of the developed heart? Harrison ('21 b) has shown that the fore-limb bud of Amblystoma is an equipotential system with axial determination in an anterior-posterior direction. Studies of this type not only add to the knowledge of the differentiation and development of the particular organ system in question, but also are of importance in connection with the problem of the basis of adult characters in the units making up the embryonic rudiments. In this respect it is of interest to determine how general the presence of equipotential systems may be in the embryo and to what extent such systems possess axial determination.

The experiments which will be described were begun in March, 1923, and were devised for the purpose of studying self-differentiation and equipotentiality, especially the latter and its relation to axial determination. Heterotopic transplantations were made for studying self-differentiation. For equipotentiality the following tests were applied: extirpation of halves of the rudiment, transplantations giving combination of halves, superposition of rudiments, and splitting of the rudiments in the midline. At similar stages of development rotations were made to ascertain if axial determination existed. In other words, a study is being made to find if there exists within the rudiment at a particular stage of development determination for differentiation in an anteroposterior, dorsoventral, or lateral direction.

The problem was undertaken at the suggestion of Prof. R. G. Harrison, and it gives me pleasure to express here my thanks to Doctor Harrison for his helpful suggestions and criticisms during the progress of the work.

MATERIAL, METHODS, AND NORMAL DEVELOPMENT

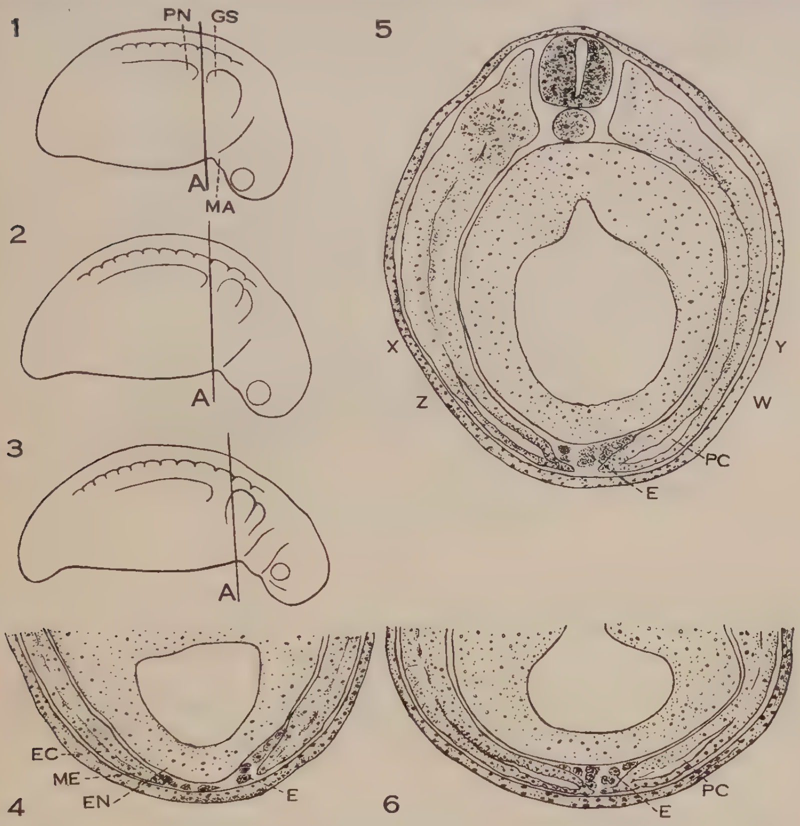
All of the experiments have been made on embryos of Amblystoma punctatum Linn. The stages have been grouped as early, standard, and late. The early stage, stages 22 and 23 (figs. 14 and 14 A), and the late stage, stages 33 and 34 (fig. 11), were used in only a small proportion of the experiments, and the type of operation at these stages will be described in the separate sections later. The majority of the operations were made at the standard stage, stages 25, 27, and 29, figs. 1 to 6.

The technique employed in the operations is similar to that already described by Harrison ('08). The area of operation in general, was as shown in figures 7 and 8. In extirpations and transplantations of anterior and posterior halves, the line dividing the tissue normally forming the organ was slightly anterior on the outline of the area for removing the entire rudiment. This is explained by the fact that in the latter case more neighboring mesoderm was removed posteriorly than anteriorly; the potency for heart development seemed to be more extensive posteriorly.

Embryos of each type of operation were preserved at varying stages of development, in order to study the effect of the operation during development as well as the later result. The embryos were preserved in the ordinary corrosive sublimate-acetic mixture. Some were studied by dissection. For softening the brittle ectoderm and rendering the embryos pliable a 5 per cent aqueous solution of nitric acid was used as described by Stone ('22). The majority of the embryos were serially sectioned and the heart studied from the sections and by means of wax reconstructions.

In order that the results of the experiments may be more clearly interpreted and in order to make clear what the degree of development is at the various stages mentioned, a brief account of the early normal development will be given.[2]

At the early operating stage, stages 22 and 23, figures 14 and 14A, sections back of the mandibular arch and throughout the region of the liver have the lateral sheets of mesoderm



Figs. 1 to 6 Standard operating stages, stages 25 to 29. *A*, plane of sections; *E*, endocardial rudiment; *EC*, ectoderm; *EN*, entoderm; *GS*, gill swelling; *MA*, mandibular arch; *ME*, mesoderm; *PC*, pericardial cavity; *PN*, pronephros; *Z* to *W*, extent of operation of type A_1, section 1 of text; *X* to *Y*, extent of operation of type A_2, section 2 of text. Figures 1, 2, and 3, stages 25, 27, and 29, lateral view; × 10. Figures 4, 5, and 6, cross-sections of stages 25, 27, and 29 at line *A* of figures 1, 2, and 3, through the anterior part of the heart rudiment; × 40.

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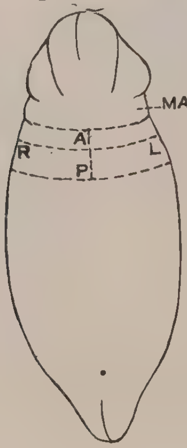
[2] Papers by Hartmann ('22, '23, '24) on Salamandra atra and axolotl give a more complete account of the early development of the heart and the vessels.

terminating in a free edge on each side. A thickening of the free edge in the region of the liver is probably the first visible evidence of rudiments of the vitelline veins.

By stage 25, beginning tail-bud stage (figs. 1 and 4), the mesoderm folds extend further ventrally, but do not reach the midline. A few cells at lower edge and to the inner side of the splanchnic mesoderm, on each side, represent the first visible rudiment of the endocardium. At stage 27 (figs. 2 and 5), embryo with thirteen to fifteen mesoderm segments and the stage at which most of the operations were made, the cells representing the rudiments of the endocardium are more ventrally placed, extending to the midline in the region ventral to the hyoid arch and the forepart of the gill swelling. At this stage the pericardial space appears between the somatic and splanchnic layers of the mesoderm on each side. The histological nature of the rudiment at stage 27 is shown in figure 9.



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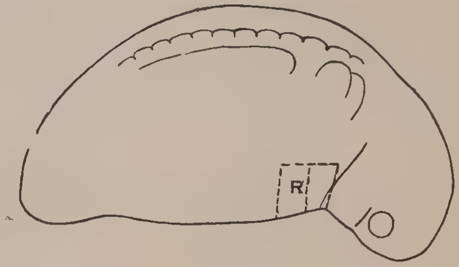

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Figs. 7 and 8 Showing operations at standard stage, in ventral and lateral view. *MA*, mandibular arch; *R*, right; *L*, left; *A*, anterior; *P*, posterior parts of area. Outer broken line indicates area of extirpation and transplantation of rudiment as a whole. Inner broken line divides area as used for operations with halves. Portion to right or left of line *AP* used for experiments with right or left halves; portions anterior or posterior to line *RL* used for experiments with anterior or posterior parts. × 15.

Cell outlines are made out with difficulty or not at all. There is no visible difference between endocardial rudiment and the mesoderm layers. Yolk globules are in the former, as in the latter, more numerous and larger than in the ectoderm, but differ little from the globules of the entoderm.

By stage 31 (fig. 10)[3] the mesoderm folds meet and join in the midventral line and form the ventral mesocardium which persists for only a short time. The endocardial cells become definitely arranged to form a hollow tube by stage 33 and a thinning of parts of the tube forming cytoplasmic strands is the first visible indication of the endothelial nature of the endocardium.

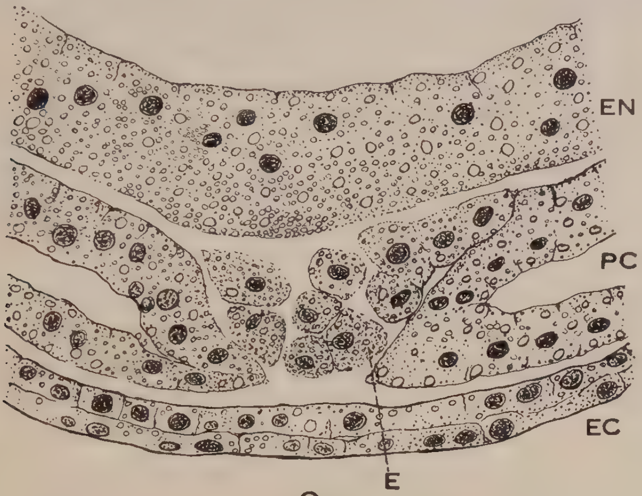

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Fig. 9 Ventral part of figure 5, stage 27, enlarged to show histological nature. *E*, endocardial rudiment; *EC*, ectoderm; *EN*, entoderm; *PC*, pericardial cavity. × 260.

Stage 34, earliest stage at which embryos show movement when stimulated, is the stage at which the myocardium begins to contract. An outline of an embryo at this stage is shown in figure 11 and the heart in figure 33. Later development consists chiefly of changes in curvature and differential growth of parts of the tube and histological changes in the walls of the tube. By stage 36 (fig. 12) the heart is very similar to that shown in figure 30; considerable curvature has taken place, and at this stage corpuscles begin to move in the vitelline veins and circulation begins in the second gill arch. By stage 37 circulation is present in all gills, and by stage 38, in the balancer also. At the latter stage (fig. 13) slight differences begin to appear in the histological nature and thickness of walls of ventricle and atrium, but the differences are minute and somewhat variable in time of appearance. Ventricular trabeculae are not clearly marked off before about stage 42 or 43.

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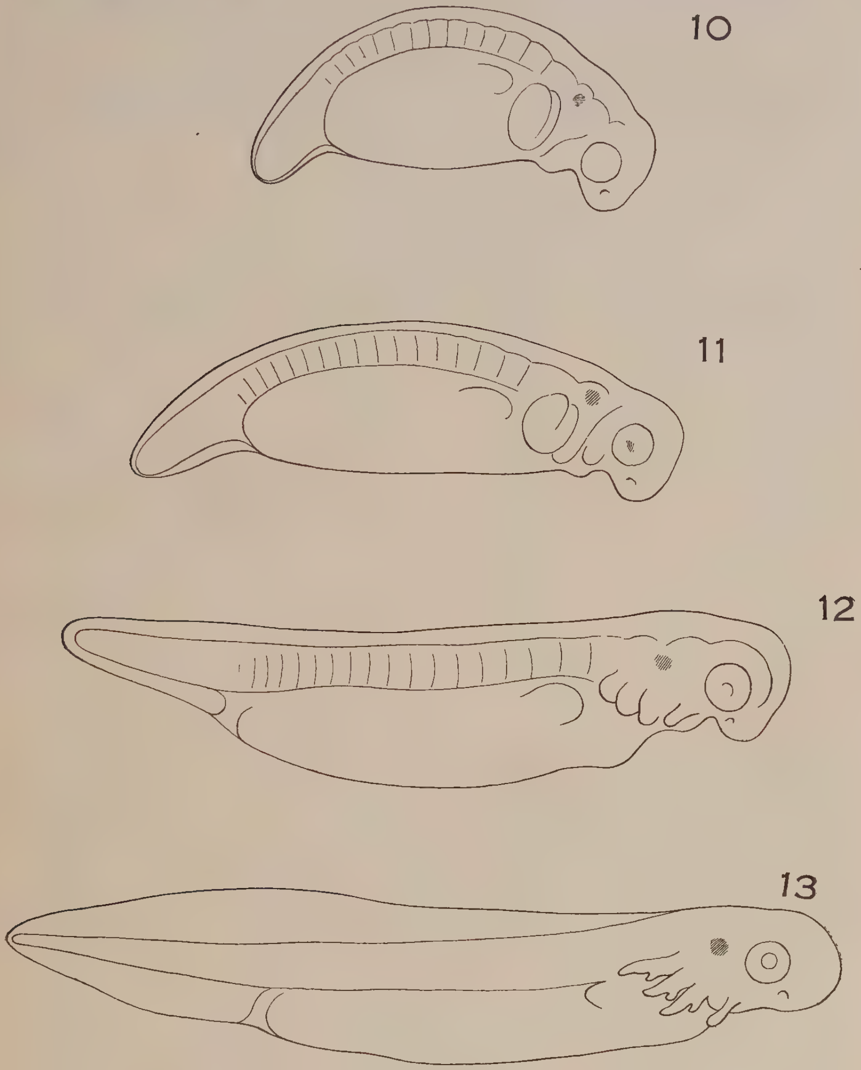
[3] The numbers referring to stages in the present paper refer to stages in the normal development of Amblystoma punctatum that have been designated by Professor Harrison and standardized by a series of drawings made by Miss L. Krause. Figures 10, 11, 12, and 13 were made by Miss Krause, and other figures of normal stages, figures 1, 2, 3, and 14, have been carefully compared with corresponding stages in the standard set of drawings.

Pulsation rates of normal hearts at various stages have been studied, and it has been found that at a constant temperature Amblystoma embryos of the same stage of development have approximately similar heart rates.[4] Operated animals with heart approximating the normal in development are therefore expected to have a pulse rate corresponding to controls of similar developmental stage. A comparison of rates was especially interesting in embryos with two hearts. For purposes of comparison, a table of normal rates has been made extending from stage 34, stage when pulsations begin, through stage 46, stage when feeding begins. It was found important to take the rate of beat at the same temperature in all cases.[5] The usual temperature in the room where the operated and control animals were kept was 19.5°C. The temperature of the water was taken in each case before and

[4] This agrees with results of Loeb and Ewald ('13), who have found that fundulus embryos of similar stage of development have similar pulsation rates at a constant temperature.

[5] In fish embryos the Q_{10} values for heart beat are similar to Q_{10} values for chemical reactions, according to Rogers ('11). In Amblystoma, the Q_{10} value at temperatures between 15° and 25°C. is 2.14, according to Laurens ('14), thus agreeing with the Van't Hoff law. Moore ('17) has found that in fundulus embryos the changes in rate are similar to changes of velocity of enzyme reactions.

after making the count and the counts always made at that temperature. Ten or more embryos were studied for each stage and average records of the rate were made for each.



Figs. 10 to 13 Four normal stages of Amblystoma punctatum. × 8. Figure 10, stage 31, stage of formation of ventral mesocardium. Figure 11, stage 34, stage of beginning pulsations. Figure 12, stage 36, stage with beginning circulation. Circulation in second gill, but usually not in first or third. Figure 13, stage 38, circulation in all gills and in balancer.

For an average record the average of three counts made at one-minute intervals was taken.

The results of the counts are given in table 1, from which it will be seen that the increase in the rate of beat was most marked: *a*) between stages 34 and 35, or just after the initiation of pulsations; *b*) between stages 36 and 37,[6] or at the time when the establishment of circulation in the gill arches is being completed, and, *c*) between stages 40 and 41. The maximum rate was reached at stages 42 and 43, when the mouth has just broken through, but feeding has not yet begun.[7] By the feeding stage, stage 46, the rate has decreased to an average of seventy-two beats per minute, and during the following stages of embryonic development the rate remains fairly constant.

TABLE 1

Rate of pulsations in controls. Beats per minute at 19.5°C.

STAGE OF DEVELOPMENT	NUMBER OF EMBRYOS STUDIED	AVERAGE RATE FOR STAGE	INCREASE (+) OR DECREASE (—) OVER PRECEDING STAGE	MAXIMUM VARIATION WITHIN STAGE	STANDARD DEVIATION
34	10	21.2		6	2.1
35	10	31.6	+10.4	6	1.8
36	10	40.8	+ 9.2	7	2.4
37	10	51.4	+10.6	9	2.7
38	19	56.8	+ 5.4	8	2.4
39	10	60.1	+ 3.3	5	2.1
40	12	65.2	+ 5.1	6	1.8
41	10	74.6	+ 9.4	11	3.4
42	10	81.2	+ 6.6	10	2.8
43	10	86.8	+ 5.6	7	2.6
44	10	83.5	— 3.3	10	3.7
45	11	77.1	— 6.4	9	2.6
46	11	72.6	— 4.5	7	2.3

---

[6] Similar to observations of Coghill and Moore ('24), who have observed marked increase of beat at time when locomotion is acquired and when gill circulation is being established.

[7] Similar to observations of Laurens ('14), who also observed that the rate decreased at the feeding stage and suggested that the decrease was correlated with influence of the vagi which become connected at about this time.

EXPERIMENTAL

A. Extirpation of heart rudiment

The results of the extirpation experiments are summarized in table 2. The operations were of the four following types: Type A_1, in which only the mesoderm, representing the actual heart rudiment, and the covering ectoderm was extirpated, that is, the mesoderm, which according to histological studies normally forms the heart, was removed. In type A_2 a much larger area was removed, extending about one-third up the side of the embryo. In operations of type Ab the area was as in A_2 and the wound was covered with ectoderm and mesoderm from a posterior ventrolateral area of an embryo of similar stage of development. Operations of type Ac were bilateral extirpations at the early stage, stages 22 to 23. Ectoderm and mesoderm were removed from each side and the wound covered as in Ab experiments.

TABLE 2

Showing results of extirpation experiments

OPERATION	NUMBER OF EXPERIMENTS		SINGLE HEART		DOUBLE HEART	VERY SMALL HEART[1]		HEART ABSENT
	Total	Positive[2]	Circulation		No circulation	Pulsations		
			Present	Absent		Weak	Absent	
A_1	60	19	3	3	11	2	0	0
A_2	50	16	0	3	3	0	5	5
Ab	24	13	0	0	0	0	1	12
Ac	47	21	0	0	0	5	5	11
Total	181	69	3	6	14	7	11	28

[1] In this group the heart was sometimes double in the Ac type of operation, but always smaller and more lateral than in cases placed under group marked 'double heart.'

[2] Excluding cases where death occurred preceding stage of beginning pulsations.

1. Development after removal of tissue normally forming heart, series A_1. The nature of the operation has already been described and the lateral extent of the area extirpated was approximately as marked by letters Z and W in figure 5. After removal of the rudiment, development was complete and circulation established in three out of nineteen positive cases. In the three embryos having single hearts but no circulation, the hearts were differentiated into sinus, atrium, ventricle, and bulbus, but were slightly smaller than normal and the differentiation was less than in controls. In a number of cases the wound did not close after the operation, due to the fact that in removing the ectoderm and mesoderm the entoderm was sometimes injured, and as a result the wound opened into the pharyngeal chamber. Animals with a wound of this nature developed a heart on each side of the wound in eleven cases.[8] The double hearts were very similar to those that will be described in a later section and further description will not be given at this point.

In none of the positive cases of series A_1 was the development of the heart completely prevented. The size of the area of operation and the fact that all mesoderm cells remaining in the wound after operation were removed insured that all of the tissue normally entering into formation of the heart had been excised. In this series, therefore, the heart developed from tissue which usually would have taken little or no part in its development.

2. Less development after removal of larger area, series A_2. The area of operation for this type of experiment is shown in figures 7 and 8. Even after removal of an area of this size development of the heart was not always prevented. The three cases shown in table 2 as having a single, approximately normal heart were operations at stage 25. In three others of the series a double organ developed, the heart on each side being small, but differentiated. In five, a single, extremely small heart developed, sometimes to one side and in other cases in the midline, depending on how quickly the wound had healed. In these no pulsations had been observed, but sections showed the presence of a small heart. Figure 36 shows a heart of an embryo of this group. By making a wax reconstruction of the organ it was found that, although small and poorly differentiated, what differentiation did exist indicated a tendency to form all parts. Anterior to the heart in this case, but not connected with it, the first two pairs of aortic arches joined in a ventral aorta forming the truncus arteriosus. Posteriorly, and also not connected to the heart, the vitelline veins and ducts of Cuvier were differentiated and united to form a sinus-like chamber. In normal development the sinus enlarges by the ducts of Cuvier and the vitelline veins joining through part of their extent, and apparently this is the part of the sinus which has developed in some of the experimental cases.

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[8] Ekman ('24 b) has used a similar method for producing double hearts, except that he removed a much smaller area and therefore only a small part of the rudiment.

The five embryos of the series in which the heart was absent were operated on at stages 28 or 29. The three in which normal development occurred had the operation done at stage 25. At stage 29, as shown in figure 6, the rudiment of the heart has become more clearly marked off from the neighboring mesoderm, and, beginning at about stage 29, this surrounding tissue apparently begins to lose its potency to form the heart, for complete extirpations at stages later than 29 were often followed by little regeneration. The potency of the neighboring tissue has not been entirely lost after stage 29, however, for two of the group having a very small heart had been operated on at stage 29 +.

3. Absence of heart when wound was covered with foreign ectoderm and mesoderm, series Ab. In operations of this type the area of extirpation was the same as in A_2, but, in addition, ectoderm and mesoderm from the flank of an embryo of similar degree of development was transplanted in place of the extirpated tissue. In thirteen positive experiments of this type pulsations were never observed and in only one case was a small heart found after sectioning. The heart in this case was normal in position, but very poorly developed. In operating the mesoderm cells of the rudiment may not have been entirely removed or, as seems more probable, the transplanted mesoderm may not have entirely blocked the mesoderm on the sides of the wound from moving in.

There was, of course, circulation in none of the embryos of the series. Animals without circulation frequently lived from two to three weeks and developed normally during the first three or four days following the operation, that is, to about stage 38, or slightly later than when circulation begins in controls. Later, development became more atypical, growth and differentiation taking place more slowly. Development of the gills was somewhat atypical in that there was less branching and less differentiation than in controls. The vascular system developed to a considerable extent in the absence of circulation.[9] The vessels became much distended and often were found to contain strands of syncytial tissue. The venous sinuses of the liver and the subintestinals were especially distended and contained many corpuscles.

4. Bilateral extirpations at stages 22 to 23, series Ac. In operations of this type (figs. 14 and 15) a piece of ectoderm and mesoderm extending dorsoventrally over approximately the middle third of the side and posteriorly from the mandibular arch for a distance equaling about two and one-half body somites was removed and ectoderm and mesoderm from the flank transplanted in its place. The wound healed quickly, and after about three hours a similar operation was made on the other side of the embryo. The operations at the early stage were made bilaterally because the rudiment of the heart is at this stage very lateral in position, the mesoderm folds ending in a free edge as shown in figure 14A.

Of twenty-one successful experiments of the series, eight embryos were preserved at stages 27, 29, 31, and 33. The remaining thirteen were kept as long as development continued and lived to stage 34, when pulsations normally begin, or later. In five of these pulsations occurred on the side,

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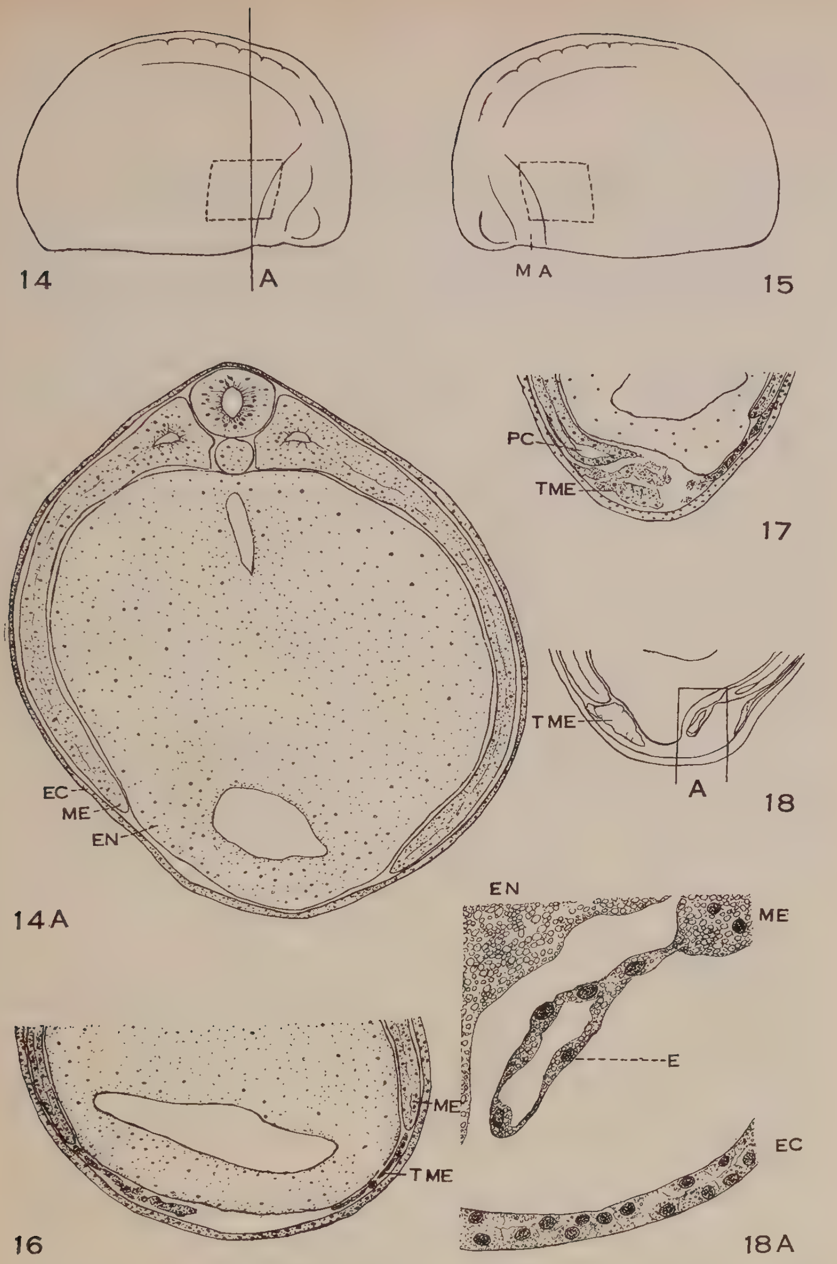
[9] Similar to results given by Knower ('07), who has described formation of vessels in frog embryos without circulation. Clark ('18), studying development of vessels in the tail of frog embryos, has also found that considerable vascular development will take place in the absence of circulation.



Figs. 14 to 18A Type of operation and results for bilateral extirpations at early stage. *E,* endocardium; *EC,* ectoderm; *EN,* entoderm; *MA,* mandibular arch; *ME,* mesoderm; *PC,* pericardial cavity; *TME,* transplanted mesoderm. Figures 14 and 15, broken line indicates area of operation at stage 22; × 15. Figure 14A, transverse section at line A, figure 14; × 40. Figure 16, transverse section through heart region at stage 27, after bilateral extirpation at stage 22. Exp. Ac 17. × 40. Figure 17, section through anterior part of heart region, stage 33, two days after bilateral extirpation. Exp. Ac 21. × 40. Figure 18, section through posterior part of heart region. Exp. Ac 21. × 40. Figure 18A, section A of figure 18 enlarged. × 200.

either on one side or in some cases on each side of the transplant. Circulation developed in none of the embryos of the series. From a study of sections it was found that the heart was entirely absent in eleven of the twenty-one cases and that ten had a very small heart on one or both sides. In some of these the heart consisted only of a pericardial space, no endocardium being present. In only one case had development gone far enough to reach the tube stage, and in that case the heart was more posterior in position than normal and had apparently developed from tissue moving in around the posterior edge of the transplant.

In embryos preserved one day after the operation the condition was usually of the nature illustrated by embryo Ac 17 (fig. 16). There was no evidence of an embryonic heart in the region where the heart rudiment is found in controls of stage 27, as seen by comparing figures 16 and 5. Embryo Ac 21, preserved at stage 33, two days after the operation, showed conditions which accounted for the fact that in some cases of this series pulsations had developed. Twelve sections back of the mandibular arch (fig. 17), the host mesoderm of the right side was found ventralward and medial between the entoderm and the transplanted mesoderm. The host mesoderm contained a small pericardial space, which was, however, much smaller than the normal of stage 33. The result shows that the area of extirpation had not included all of the mesoderm with potency for heart formation and that this part of the mesoderm, after moving ventralward between the transplant and the entoderm, had formed the rudiment of a small heart.

In embryo Ac 21, several sections posterior to the one shown in figure 17, there was a pericardial space on the left side. In connection with the mesoderm of the left side there was a group of cells resembling endocardium in appearance (fig. 18A). The origin of endocardial cells is difficult to determine by studies of series of stages in normal development, and consequently there has been some doubt concerning the origin of the endocardium in the urodeles. The fact that in embryo

Ac 21 and in two others of the series endocardial cells were seen attached to mesoderm indicates a mesodermal origin. Also, in several of the embryos of the series endocardium was entirely lacking, in spite of the fact that none of the tissue in the midline had been removed and that the entoderm had not been injured. Such results cannot well be harmonized with the view that the origin of the endocardium is in the entoderm.

B. Transplantation of rudiments

5. Orthotopic transplantations, antero-anterior orientation. Experiments of this type were made as controls in connection with the rotation of transplants. Ectoderm and mesoderm were transplanted with normal orientation, the area of operation being the same as in Ab series of extirpation experiments, as shown in figure 7. Of the ten positive cases of this type, circulation developed in seven. The three embryos which did not develop circulation had pulsations of normal direction, but slower than normal, and study of sections showed that the heart was differentiated into all parts, but smaller than in controls. The results for this series and for the other orientations and locations are summarized in table 3.

6. Orthotopic transplantations, antero-posterior orientation. In operations of this kind the transplant was rotated 180° about the dorsoventral axis, placing the originally posterior part anteriorly and vice versa. In all of the thirty-seven positive experiments a pulsating heart developed, but circulation never occurred. In one case two separate regions of pulsation were observed; one at the anterior edge of the transplant due to regeneration from the host and the other posteriorly due to development from the transplant. In the majority of cases the pulsations were of an approximately normal rate, being retarded only about one stage; that is, when the embryo was at stage 38 the heart pulsated at a rate similar to controls of stage 37. In some of the embryos the pulsations were weak and extended over only a very small area; in such cases the direction of beat was difficult to determine. However, in twenty-seven cases the direction could be determined and was found to be reversed, the contraction beginning anteriorly where the bulbus would normally have been and ending posteriorly.[10] As circulation never developed, the embryos differentiated for only a short time; likewise the heart developed through a few stages only, and in the cases studied never exceeded in rate of beat the normal rate of stage 38.

TABLE 3

Showing results of transplantations of rudiments

OPERATION	NUMBER OF EXPERIMENTS		HEART SINGLE BEAT				HEART DOUBLE BEAT	
	Total	Positive	Normal		Reversed	Weak. indefinite	Reversed	Indefinite
			Circulation		No circulation			
			Present	Absent				
Orthotopic, antero-anterior	16	10	7	3	0	0	0	0
Orthotopic, anteroposterior	123	37	0	0	27	9	1[1]	0
Orthotopic, bilateral	43	8	0	0	1	0	4	3
Heterotopic, antero-anterior	11	6	0	4	0	2	0	0
Heterotopic, anteroposterior	26	14	0	0	10[2]	4	0	0
Total	210	75	7	7	38	15	5	3

[1] Pulsations in anterior part in normal direction and in posterior part of transplant reversed; former from host, latter from transplant.

[2] Slight circulation in one case.

The morphology of the rotated hearts was somewhat variable and often difficult to interpret because of the poor degree of differentiation. In the majority of cases it was as in the following examples. Embryo F 106, preserved at stage 36, two days after the operation, was studied by dissection and found to be differentiated into four parts as shown in figure 19. A small mass of tissue attached to the anterior edge of the heart resembled in appearance the rudiment of the liver and has been interpreted as a transplanted fragment of that organ. The heart consisted of an anterior portion partly divided into two parts, the part adjacent to the transplanted liver tissue forming a small cap-like portion, the sinus, on the larger more rounded part, the atrium. Posteriorly the heart had a relatively well-developed ventricular portion, but the bulbus cordis was poorly marked off, being only a small and solid tube-like extension from the ventricle toward the left side of the liver. The atrial and ventricular portions were fused dorsally to the entoderm of the gut, the other parts were free dorsally as well as ventrally and laterally in the pericardial space. The morphology of the dissected heart corresponded to the direction of pulsation previously observed. Another example of the series (embryo F 116, fig. 20) had the heart more clearly differentiated than F 106. The sinus and bulbus were developed more in proportion to the other parts. In each case the heart was reversed in the sense that the parts were oppositely placed with respect to the position characteristic for controls.

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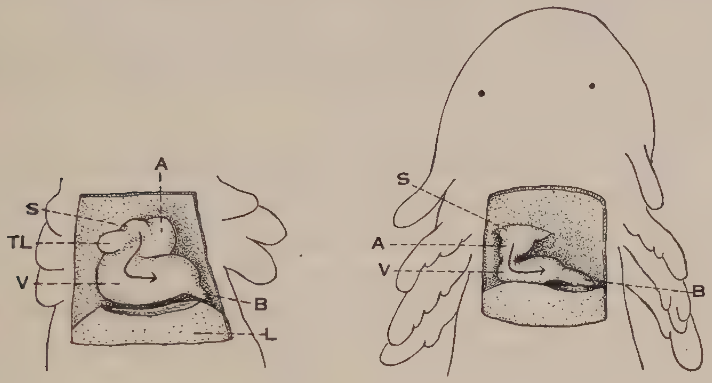
[10] Gowanloch ('22) has described for fish embryos, after treatment with chemical or physical agents a physiological reversal of pulsations, the beat being from anterior to posterior; the morphology, however, being normal and not reversed as in the experiments here described.



19 20

Figs. 19 and 20 Orthotopic transplantations, anteroposterior orientation. Ectoderm, parietal pericardium and small part of liver dissected away. A, atrium; *B*, bulbus; *S*, sinus; *TL*, fragment of transplanted liver; *V*, ventricle. Figure 19, embryo at stage 36, two days after the operation. Exp. F 106; × 20. Figure 20, embryo at stage 38, four days after the operation. Exp. F 116; × 20.

Some of the more abnormally developed cases of the series which were more difficult to understand were studied by wax reconstructions. Figure 37 shows one of these from an embryo preserved five days after the operation. Differentiation was not marked, but there was evidence of four parts, the bulbus, however, being especially poorly differentiated from the ventricle.

Concerning the form of the heart, it was interesting to note that in this series the type of curvature was not that of a rotated normal, but variable and often approximately of a non-rotated normal. This is evident from comparison of figures 19, 20, and 37 with figure 35, a photograph of a wax reconstruction of a normal heart of only a slightly later stage of development.

7. Orthotopic, bilateral with anteroposterior orientation. The operations were made at stages 22 and 23, when the mesoderm ends in a free edge on each side. A piece of ectoderm and mesoderm was removed from one side of the embryo from an area just back of the mandibular arch, as shown in figures 14 and 15, and as described for series Ac in section 4. A similar piece of tissue was transplanted from an embryo of the same stage of development and rotated 180°, placing the originally anterior part posteriorly. After about four hours healing had occurred, and a similar operation was done on the other side. The mortality after bilateral transplantations was relatively high, and only eight out of forty-three operations were successful. Sections of embryos preserved one and two days after the operation showed various degrees of fusion of the transplanted and host mesoderm along the dorsal side of the transplant and the transplanted mesoderm had moved ventrally and medially, but not as much so as in controls, that is, the mesoderm folds did not extend to the midline as early in development, and as a result double hearts with varying degree of fusion developed in seven out of eight positive experiments. In all embryos in which the direction of beat could be determined it was found to be reversed.

The amount of differentiation and the form of curvature were somewhat variable, but were, in general, as in embryo Acf 38, which was preserved at stage 39, nine days after the operation. The heart in this case was studied by reconstruction (fig. 38), and found to be double and shaped in a way corresponding to the course of double pulsations earlier observed. The degree of differentiation was less than normal and the curvature was somewhat atypical. Differentiation, especially of the left heart, was sufficient to show that reversal had occurred, the anterior part resembling in shape a small sinus and atrium rather than a bulbus, and the posterior part being clearly ventricle and bulbus. The right heart was not clearly differentiated and was small, but approximated in form a mirror image of the left.

8. Heterotopic transplantations, antero-anterior orientation. Heterotopic transplantations,[11] that is, transplantations to other than the normal position, were made to a posterior ventral region of the embryo. Ectoderm and mesoderm, the latter being very thin or absent in the region of operation at stage 27, were removed and the heart rudiment of another animal implanted with normal orientation. Pulsations were observed to begin in the transplant at about the same time as in normal position. The direction of pulsations was normal, that is, from posterior to anterior.

With respect to rate of beat, an interesting result was observed in embryo E 4. Four days after the operation (embryo at stage 36 +), the rate was forty-four beats per minute in the host heart and forty-seven per minute in the transplanted heart, in spite of the fact that the host heart was much larger than the transplanted heart as shown in figures 21 and 22.

Although the transplanted hearts were smaller than the host hearts, were less clearly differentiated than in controls, and often had the bulbus and sinus relatively small, they did show differentiation into all parts, when there was sufficient differentiation to determine any part. As the transplant consisted of only ectoderm and mesoderm of the heart rudiment, all neighboring organs being excluded in some of the cases, the fact that the transplanted mesoderm developed into a differentiated pulsating heart in a heterotopic position shows the potency for self-differentiation in the heart rudiment.

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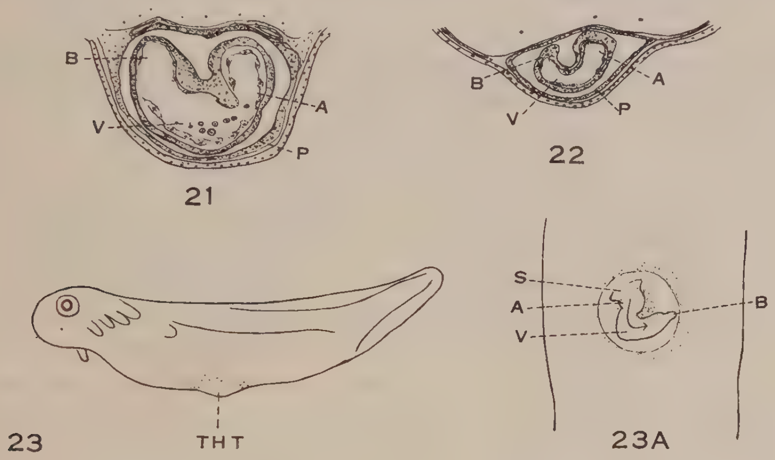
[11] This series of experiments is somewhat similar to the transplantation experiments described by Stöhr ('24 c, '24 d), except that in the present experiments the operation included much less of the neighboring tissue.

9. Heterotopic transplantations, anteroposterior orientation. The heart rudiment was transplanted to a median ventral position posterior to normal and rotated 180° about the dorsoventral axis, thus placing the originally posterior part anteriorly. The development was, in a general way, the same as described for heterotopic non-rotated transplants, in that the heart developed in a pericardial chamber in a cavity formed by a depression of the yolk and a bulge of the ectoderm. In some cases the pericardium was incomplete dorsally. Also, as in the non-rotated type, the heart developed according to its original orientation, so that after rotation pulsations were from anterior to posterior. In only one case did circulation through the transplanted heart occur. In this embryo the heart was connected with small vessels of the yolk and a few corpuscles were seen to pass through the transplanted heart at each beat. The direction was at first according to the original axis of the transplant, but shifted slightly and became more at right angles to the long axis of the embryo.

Differentiation was not as clear as in the normal host heart, but showed that four parts were formed in those cases in which there was any degree of differentiation of any one part. Embryo Ea 12 is a typical example of the series. The drawings (figs. 23 and 23A) were made after clearing the animal in cedar oil, and study of sections later verified the correctness of the interpretation. In a few cases, as in this one, the form could be made out clearly through the transparent ectoderm and dissections or reconstructions were unnecessary. The bulbus and sinus were relatively small. In curvature there was a resemblance to the heterotopic non-rotated transplants and also similarity to the normal; in other words, the curvature differed from what would have been found had development occurred strictly in accordance to rotation. Embryo Ea 6, as an example of one of the more atypical cases, was studied by wax reconstruction (fig. 39). The differentiation was not so clear as in embryo Ea 12, but all parts were present and arranged in a sequence corresponding to the original postero-anterior axis of the transplant. The curvature was atypical, being almost entirely in a dorsoventral direction.



Figs. 21 to 23A Heterotopic transplantations. *A*, atrium; *B*, bulbus; *P*, pericardium; *S*, sinus; *THT*, transplanted heart; *V*, ventricle. Figure 21, transverse section through heart of host, stage 36 +, embryo of exp. E 4. × 50. Figure 22, section through transplanted heart, antero-anterior orientation. Exp. E 4. × 50. Figure 23, showing position of transplanted heart, antero-posterior orientation. Lateral view. Exp. Ea 12. × 8. Figure 23A, same as above, after clearing in cedar oil. Ventral view. Exp. Ea 12. × 20.

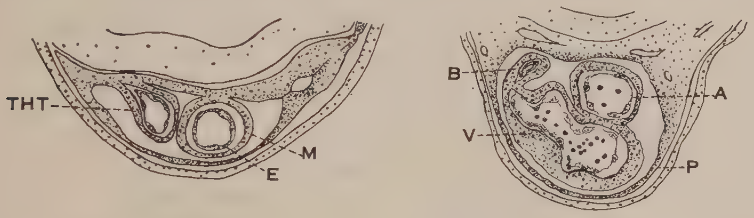
C. Effect of superposing heart rudiments

An organ system which is equipotential should be capable not only of developing two or more organs from the one embryonic rudiment, but also of forming one organ from two rudiments. In order to make a study of this question, heart rudiments were superimposed. The operations were made at the standard stage and the area was the same as in the extirpation and transplantation experiments. Ectoderm covering the area was removed and ectoderm and mesoderm of the operation area transplanted from an embryo of similar stage of development. Operations of this type were made with normal orientation and with rotation of 180° about the dorsoventral axis.

10. Superposed rudiments, antero-anterior orientation. Sixteen operations of this type were made, ten of which were positive. Pulsations in each of the ten cases were normal in direction and all of the embryos which were kept until the normal circulation stage developed gill circulation. The heart beat could be more easily studied than in controls, for it was more pronounced, but the rate was approximately normal. Embryo S 13, preserved three weeks after the operation, was sectioned and found to have the heart normally differentiated and of normal size. Sections of other embryos at earlier stages of development indicated that the heart was larger than in controls during the early stages; the myocardial and pericardial layers were thicker than normal and the tube was of larger diameter. In later stages these differences were not found.

In nine of the ten positive experiments the heart was single; in only one case was reduplication observed. A transverse section through the heart in this case is shown in figure 24. The transplanted heart was to the right side of the host heart and attached to the dorsal side of the pericardial chamber. The two were not in contact except posteriorly. The failure of the two rudiments to unite to form one organ was doubtless due to the transplant's having been placed to one side. The operation in this case had been made at stage 29, when the pericardial spaces in the mesoderm fold have definitely formed, and placing the transplant to one side caused the groups of endocardial cells for the two rudiments to be side by side rather than directly superposed, and each rudiment continued its independent differentiation. The fact that in nine out of ten positive experiments the result was as shown in figure 25, that is, one organ formed from two rudiments, indicates that the system was equipotential at the stages of operation.

11. Superposed rudiments, anteroposterior orientation. Eight operations of this type were made, five of which were positive. Pulsations developed in each of the embryos, but circulation was never observed. The pulsations were atypical with respect to the direction of the beat. An attempt to pulsate in a postero-anterior direction appeared to be blocked and there occurred a more or less simultaneous pulsation of all parts; the direction being in a ventrodorsal direction rather than in the typical postero-anterior sequence.



24 25

Figs. 24 and 25 Results of superimposing rudiments, antero-anterior orientation. *A*, atrium; *B*, bulbus; *E*, endocardium; *M*, myocardium; *P*, pericardium; *THT*, heart from superposed rudiment. Figure 24, showing failure of two to combine to form one organ; atypical result. Exp. S 6. × 50. Figure 25, typical result. Heart approximately normal, except slightly larger. Four days after the operation. Exp. S 14. × 50.

Sections showed that fusion to a varying degree had taken place. Embryo Sa 3 was a typical case. A separate tube extended from the posterior ventral part of the bulbus posteriorly. In the region of the ventricle and atrium it was in contact with and partly open into these parts of the host heart. The ventral tube, the heart from the transplant, was only slightly differentiated, but sufficiently to show that the sinus and atrium were anterior. The postero-anterior axis had been determined before the operation and the parts which had developed in accordance with their original relations were thus disharmonic with respect to the host heart and had failed to fuse to as great a degree as in the superposed non-rotated transplants. Also the determination caused the pulsations of the two rudiments to oppose and thus interfere with circulation.

D. Splitting of the rudiment into right and left halves

12. Extirpation of a median area for the purpose of dividing the rudiment. The operation consisted in removing a median part of the rudiment and transplanting in its place a piece of ectoderm and mesoderm from the flank of an embryo of similar stage of development. In extirpation experiments previously described, when the wound healed slowly, two hearts were formed and likewise in the bilateral extirpations a few cases had pulsations on each side. As most of the rudiment had been removed, the hearts which developed were very small. In the present series less of the rudiment was removed and development was more typical.

The first operations made included only a very narrow piece of tissue. Eighteen operations of this type were made, fifteen of which were positive. In one embryo pulsations developed on only one side and in three the heart was single and normal to the degree of having circulation. In eleven the pulsations began double, but in seven of these the beat later became single. In later operations a larger area was removed and a larger proportion of double hearts developed. Of eleven positive experiments, six embryos were preserved at early stages to study the process of development and the remaining five developed double pulsations. In the various types described a total of thirty-three embryos with double hearts were produced.

The pulsation rates were studied and typical examples are given in table 4. In Ad 14 an interesting point was that the beat which began double and at different rate for the two sides became much less in rate after becoming single and central. This was the result only when beating in unison and partial fusion took place at stages after 37. In earlier stages, as in embryo Ad 3, the rate which obtained when the heart was double sometimes persisted after fusion to a single organ and a functional heart with circulation developed. In later stages when the two hearts came into contact the rate always became less and finally ceased, the previous development and differentiation having reached such degree as to bring about an interference when the two beating at different rates came into contact.

Study of sections showed that all embryos with different rates had the two hearts separate in the region of the sinus and atrium. Embryos with pulsations single sometimes had reduplications, but the doubling existed only in the fore part, any separation at the caudal part always causing separate pulsations.

TABLE 4

Pulsation rates in embryos having two hearts

EMBRYO	DAYS AFTER OPERATION	STAGE	PULSATION RATE AT 19.5°C.		
			Right	Left	Central
Ad 3	2	34+	20	25	
	3	36	31	31	
	5	37			40
	12	42			62
Ad 8	2	35	Irregular	32	
	3	36+	Irregular	41	
	4	37	Absent	45	
Ad 14	2	35+	41	33	
	3	37	52	43	
	4	38	49	49	
	5	38+			27
Ae 24	3	35	31	35	
	8	39	55	51	
	12	41	68	60	
	16	43	71	63	

The morphology of the double hearts was usually as is shown in figure 26, for embryo Ae 16. The curvature was more in the dorsoventral direction than normal. In form, the left was approximately as in controls, while the right showed situs inversus. A similar result was observed in all embryos in which development of two hearts occurred, that is, situs inversus was always present on the right side. With respect to the pericardium, there was much variation. Some embryos had a single pericardial chamber for the two hearts, some had the cavity completely double, others showed union of the cavities of varying degree.

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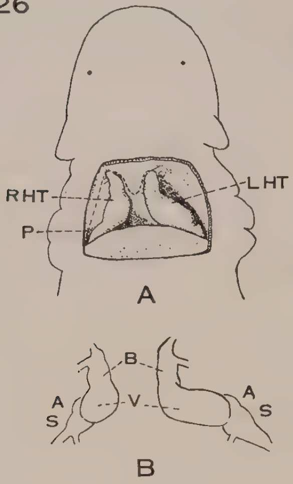

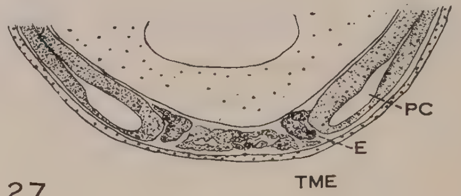

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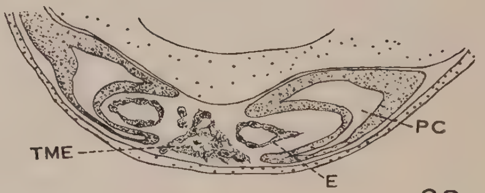

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Figs. 26 to 28 Effect of splitting rudiment into right and left parts. *A*, atrium; *B*, bulbus; *E*, endocardium; *LHT*, left heart; *P*, pericardium; *PC*, pericardial cavity; *RHT*, right heart; *S*, sinus; *TME*, transplanted mesoderm; *V*, ventricle. Figure 26A, ectoderm and ventral part of pericardium removed. Hearts in position of development, three days after the operation, stage 35. Exp. Ae 16. × 20. Figure 26 B, same after further dissection and slight rotation of hearts toward median plane of embryo. Exp. Ae 16. × 20. Figure 27, stage in development of double hearts. One day after operation. Stage 32. Exp. Ae 19. × 50. Figure 28, later stage in development of double hearts. Two days after operation. Stage 33 +. Exp. Ae 22. × 50.

Circulation while the two hearts were entirely separate and pulsating at different rates occurred in only one embryo of the series. In most cases the development was too far lateral and there was atypical connection or absence of connection with the aortic arches. In embryo Ae 24 (fig. 34), circulation began through the right heart at stage 37. Circulation was normal through the gills on the right side and weak on the left, probably the result of slight connections in the region of the truncus arteriosus. At stage 38 there was circulation through each heart, the two beating at different rates. The circulation and separate pulsations continued until stage 43, sixteen days after the operation, when the rate became irregular and weak and circulation ceased. The embryo was sectioned and found to have the bulbi united in a common truncus arteriosus; all other parts were completely double and the pericardial cavity was double caudally.

The process of development of double hearts is shown in figures 27 and 28.[12] The pericardial space and endocardial cells formed a heart on each side of the embryo instead of uniting to form one as in normal development. Only one mesocardium developed, it being dorsomedian in position and the ventral one being absent as a result of the development from one mesoderm fold rather than by union as in normal development.

E. Extirpation of parts of rudiment

13. Extirpation of anterior part. As stated in the section on normal development, the heart rudiment is very short in an anteroposterior direction, at the stages of operation, and consequently extirpation of anterior or posterior halves is more difficult than similar operations for right or left halves and the part removed more likely to be only an approximate of the half. The area of operation is shown in figure 7. After extirpation of the half, the wound was covered with foreign ectoderm and mesoderm to prevent regeneration from the edge of the area. The results for this series and for all of the extirpation of parts of rudiment experiments are summarized in table 5.

The number of positive cases in this series was small, but the results conclusively showed that parts of the rudiment which normally would have formed only the posterior half had formed all parts. The transplanted ectoderm and mesoderm acted as a block, preventing union of heart and aortic arches. Consequently, there was no circulation and development and differentiation continued normally for only a short time. The typical form and degree of development reached in most cases of the series is illustrated by embryo O 6 (fig. 29). Embryo O 6 was particularly of interest because of the fact that there was evidence for believing that the heart had developed from less than a posterior half of the rudiment. Dissection showed that two hearts had developed: an anterior one, which was small and undifferentiated, and a posterior one, which was more fully developed. The fact that enough of the anterior half of the rudiment had been left to regenerate a small heart and that an area of supposedly half the heart rudiment had been removed posteriorly made it clear that the posterior heart had developed from tissue which normally would have formed less than half of the organ, yet this part had developed a whole. The space between the anterior and posterior hearts indicated in the figure does not represent the relative size of the area of operation, for during development the hearts grew partly into the area of the transplanted foreign mesoderm, but never to sufficient extent to form a tube properly connected with aortic arches and capable of circulation.

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[12] Similar to development of double hearts described by Ekman ('24 b) for Bombinator embryos.

TABLE 5

Showing results of extirpation of parts

PART EXTIRPATED	NUMBER OF OPERATIONS		HEART OF NORMAL PARTS		POORLY DIFFERENTIATED	PARTLY REGENERATED	SLIGHT OR NO REGENERATION
	Total	Positive	Circulation	No circulation			
Anterior half, standard stage	11	6	0	4	2	0	0
Right half, standard stage	33	21	2	16	3	0	0
Central part, late stage	13	10	0	2	0	3	5
Anterior part, late stage	8	5	0	0	0	1	4
Posterior part, late stage	12	8	1	2	0	4	3
Right half, late stage	12	10	3	0	0	2	5
Left half, late stage	9	6	3	1	0	2	0
Total	98	66	9	25	5	10	17

14. Extirpations of right or left halves. A right half of the heart rudiment was removed, the extent and position of the area being as indicated in figure 7. After extirpation the wound was covered with ectoderm and mesoderm from the flank of an embryo of a similar stage of development in order to block regeneration from the edge of the wound. Of the twenty-one positive experiments made, eighteen embryos developed a heart of all parts, while three did not show sufficient differentiation for a determination of parts to be made. Embryo R 16 may be taken as a typical example of the series. Dissection showed that the heart was differentiated into sinus, atrium, ventricle, and bulbus as shown in figure 30. The heart was located more to the left side than normal and the curvature in the posterior part was more in the vertical direction than in controls.

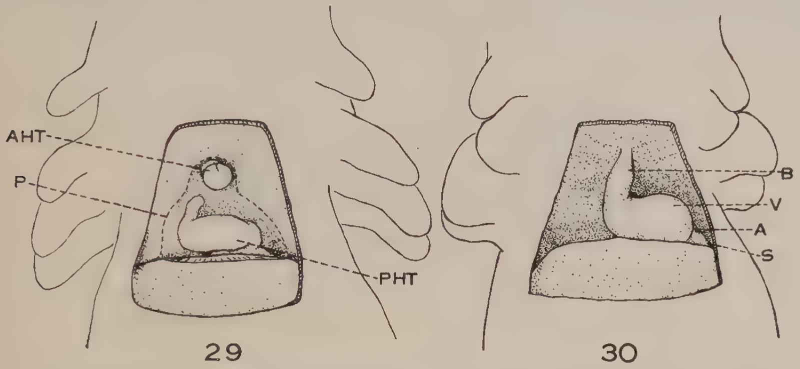

Fig. 29 Result of removal of anterior part of rudiment. *AHT*, development of fragmentary heart at anterior edge; *PHT*, posterior heart of all parts; *P*, pericardium. Ectoderm and ventral part of pericardium dissected away. Exp. O 6. × 30.

Fig. 30 Result of removal of right half. Dissected at stage 35 +. *A*, atrium; *B*, bulbus; *S*, sinus; *V*, ventricle. Exp. R 16. × 30.

Circulation developed in only two of the embryos of the series and study of sections showed that often the bulbus was not normally connected with the aortic arches, being lateral to the union of the arches into the truncus arteriosus. This may have resulted from the fact that the operation often included slightly more than half of the rudiment, and accordingly, extending over the midline, interrupted the continuity of the endocardial part of the rudiment where it extended into the ventral aortic rudiments. Another factor in causing the heart to be more lateral was its method of development, which occurred by an inpushing and growth around the endocardium from one mesoderm fold, and not by union of the two sides as in normal development. A stage in the development is shown in figure 31, a cross-section of an embryo preserved two days after the operation. By this method of development the heart was more lateral in position.

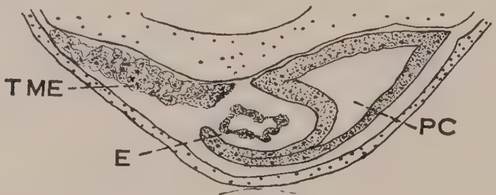

Fig. 31 Showing development after removal of right half. Section through the posterior part of heart, two days after the operation. Stage 34 +. × 70.

15. Extirpation of parts at late stage. In order to determine how late in development ability for regeneration existed, parts were removed at stages just preceding and at the beginning of pulsations; that is, at stages 33 and 34. The heart at these stages and the types of operations are shown in figures 32 and 33. In operating, the ectoderm over the pericardial chamber was turned to one side, an incision made in the parietal pericardium, and a part of the tube removed. The ectoderm was then turned back into position and the wound healed rapidly. The operations made at the late stage were of five types: removal of a large central part of the tube,[13] removal of anterior part, of posterior part, of right half, and of left half. The results are summarized in table 5.

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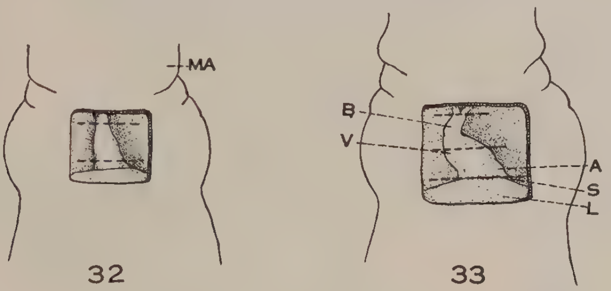
[13] This operation was similar to a type described by Ekman ('21) except that a slightly larger portion of the tube was removed, thus including all of what would have developed into the ventricle.

In the first type of operation, indicated in figure 32 for stage 33 and similarly done in other stages, the part extirpated included the posterior part of the bulbus, all of the ventricle and the anterior part of the atrium. Circulation did not develop in any of the embryos of the series. Pulsations occurred separately at the anterior and posterior parts in some, and in such cases the rate was faster in the sinus portion than at the bulbus and rhythmical at the former, but irregular at the latter position. From sections it was found that in two animals all parts of the heart were present, though somewhat atypical. In three others part regeneration only occurred; that is, the anterior and posterior parts had not become connected to form a tube, but a small ventricle had formed, being joined to the anterior part of the atrium. In five embryos of the series regeneration was practically absent, the sinus being normal, atrium small and ventricle absent. In each of these cases the operation had been made at stage 34 or later, that is, at the time when pulsations begin.



Figs. 32 and 33 Operations at late stage. Figure 32, stage 33. *MA*, mandibular arch. Broken line indicates point of incisions for extirpation of central part of tube. × 20. Figure 33, stage 34. Broken line indicates points of incision for removing anterior or posterior parts. *A*, atrium; *B*, bulbus; L, liver; *S*, sinus; *V*, ventricle. × 20.

To study regeneration after removal of a smaller amount of the tube, extirpations of approximately one half of the heart were made. Extirpation of an anterior half included the anterior part of the ventricle and all of the bulbus; extirpation of a posterior half included the posterior part of the ventricle and all of the atrium. The results were very similar to the results after removal of a central part; when operations were made at stages 33 or 33 +, regeneration was more marked than when operations were made at stage 34 or later.

The operation of removing a right or left half consisted in making a longitudinal incision in the tube from the cephalic end of the bulbus to the central part of the sinus and extirpation of the half by an incision half-way through the tube transversely at the anterior and posterior limits of the longitudinal cut. After removal of right or left halves, the proportion developing circulation was greater than after removal of anterior or posterior halves and the formation of a complete tube was found to be possible after operations at a slightly later stage, occurring as late as stage 34 in some cases. In general, however, there was little regeneration when the operation was made after pulsations had begun, and in this respect there was agreement in all of the types of operations made at the late stage.

Thus, in all types of operations at the late stage removal of a part was followed by complete formation of the heart in some embryos of each type by a process of regeneration; that is, after removal of a part there was rapid growth at the cut surface and from the newly formed tissue the parts

Fig. 34 Photograph of embryo Ae 24, showing position of two hearts. Ventral view. × 5.

Fig. 35 Reconstruction of normal heart, stage 40, showing aortic arches of the truncus arteriosus and veins entering sinus. Ventral view. × 100.

Fig. 36 Reconstruction of small heart developed after extirpation of the rudiment when the wound was not covered. Exp. A_2 126. Ventral view. × 100.

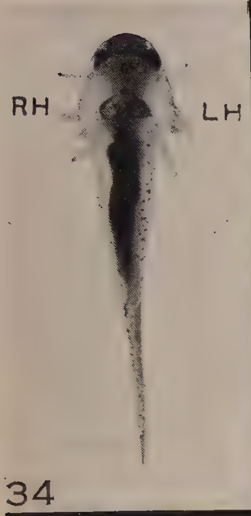
Fig. 37 Reconstruction showing form of one of the orthotopic transplants with anteroposterior orientation. Ventral view. Exp. F 123. × 100.

Fig. 38 Reconstruction of double heart after bilateral transplantation with rotation at early stage. Exp. Acf 38. Ventral view. × 100.

Fig. 39 Reconstruction showing heart after heterotopic transplantation with anteroposterior orientation. Exp. Ea 6. Seen from left side. × 100.

ABBREVIATIONS

AT, atrium
B, bulbus
LH, placed opposite left heart
RH, placed opposite right heart
S, sinus
V, ventricle



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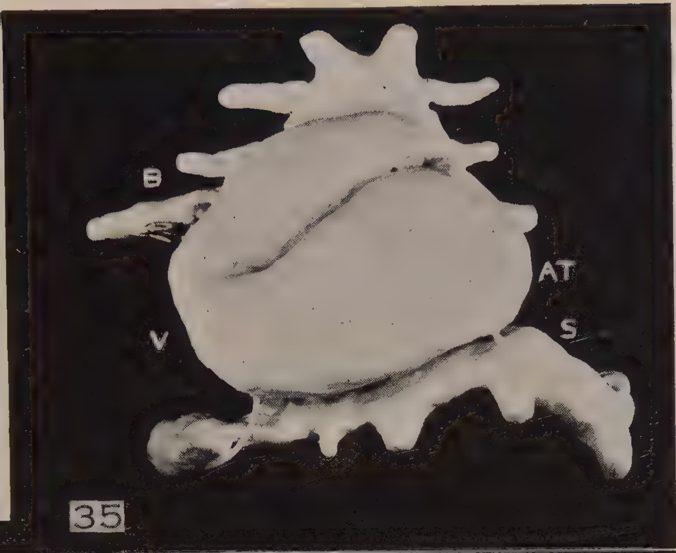

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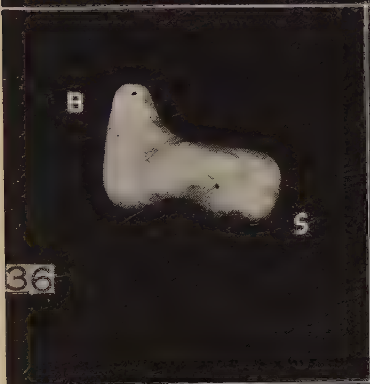

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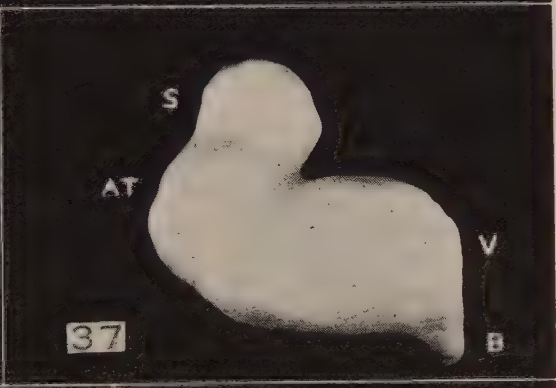

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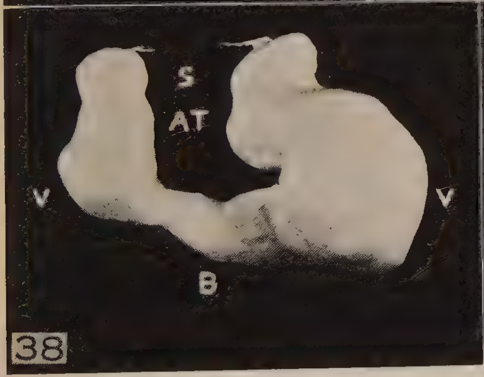

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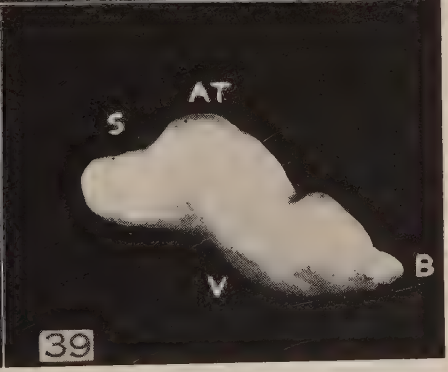

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which had been removed were differentiated. However, there was reason for believing that in some cases parts of the tube destined to form one region were partially changed over directly into another. This occurred in cases when the cut ends of the tube joined quickly after the operation. After an operation in the region of the heart the nape bend of the embryo was increased and the cut ends of the tube were brought together soon after the completion of the operation. In some of these the heart which developed was found to be somewhat smaller than normal in all parts, the parts being typical as regards relative size. Accordingly, in these some of the tissue which would have formed bulbus, for example, had partly entered into the formation of the ventricle. In general, the heart rudiment lost the qualities of an harmonic equipotential system between stages 30 and 33. The power of regeneration was marked as late as stage 33, but decreased rapidly thereafter, being practically absent by stage 34 +.

F. Transplantation of parts

To determine if two halves when placed together would develop a normal whole or whether each would develop the part which it would have formed if left in normal position, transplantations have been made in such a way as to give various combinations of halves. The results are summarized in table 6.

17. Transplantations giving two anterior parts. In operations of this type, the posterior half of the rudiment was removed and an anterior half from an embryo of similar stage of development was put in its place with normal orientation. The positive cases given in the table exclude all embryos which did not live until the stage when pulsations normally begin, and also all embryos in which the transplant was sloughed off or improperly placed. In three out of seven positive experiments, hearts developed normally to the extent of having circulation, and in two which lacked circulation small hearts showing all parts developed. In two others pulsations had been weak, and in each of these the heart was found to be very small and poorly differentiated.

In this type of operation and in types to be described in the following sections there exists the possibility that the transplant has had no part in forming the organ, development having occurred entirely from the half which had not been extirpated. There is, however, reason for believing that the transplant entered into the development, for, if it did not, then the mesoderm of the transplant would have had an effect similar to that of the foreign mesoderm which was transplanted in the experiments of extirpation of the anterior half. In that series, table 5, circulation never developed, the heart being in all cases unconnected with the aortic arches. In the present series circulation occurred in 50 per cent of the positive experiments.

TABLE 6

Summary of results of transplantation of parts

OPERATION, COMBINATION OF HALVES	NUMBER OF EXPERIMENTS		HEART NORMAL		HEART SMALL, DIFFERENTIATION POOR	PARTLY REDUPLICATED
	Total	Positive	Circulation			
			Present	Absent		
Two anterior	11	7	3	2	2	0
Two posterior........	13	8	4	3	1	0
Two right or two left	39	27	7	14[1]	4	2[2]
Totals	63	42	14	19	7	2

[1] Ten of this number not kept as late as stage of circulation. Of embryos kept to circulation stage, circulation was observed in eight out of fourteen.

[2] One of these had only very slight reduplication and had circulation.

18. Transplantations uniting two posterior parts. In operations of this type an anterior half was removed and a posterior half was transplanted in its place. Of eight positive experiments circulation developed in four cases, as shown in table 6. Sections of animals with circulation showed normal hearts. Sections of embryos without circulation showed some abnormalities, the heart usually being small and atypical in all parts rather than in any particular one, and it was found that parts of the tube sometimes failed to develop a continuous chamber. In considering cases of this type, the fact must not be overlooked that the heart rudiment is of very plastic nature and abnormalities are to be expected from operation effects. The fact that 50 per cent of the cases were functional to the point of developing circulation shows that two posterior parts can form a normal organ, and in this sense are totipotent.

19. Transplantations giving two right or two left halves. To combine two right halves a left half of the heart rudiment was removed as indicated in figure 7, and a right half from an embryo of similar stage of development was transplanted in its place with no rotation. Similar operations were made for the left side, producing combinations of two left halves. The results of the two types of operations are combined in table 6, being similar in both.

Hearts which were normal to the extent of having circulation developed in eight embryos out of a total of twenty-seven positive experiments. Of the twenty-seven, only fourteen were kept until stage 37 or later. As the operation usually slowed up the development of the heart about one stage, circulation should not be expected in the operated animals before stage 37, and accordingly the proportion having circulation was seven out of fourteen, rather than seven out of twenty-one. One of the embryos which had circulation was found to have partial reduplication of bulbus and ventricle; all other embryos having circulation were found to have normal hearts. Embryos kept to stage 37 or later and lacking circulation were found in a few cases to possess very small undifferentiated hearts; in other cases the heart was differentiated into all parts, though somewhat atypical. Atypical conditions noted were not specific in the sense that they could be correlated with the type of operation as an indication of a mosaic nature of the rudiment. The results of the series, including combinations of two posterior, two anterior, or two right or left halves, indicated equipotentiality in the sense that two similar halves of the rudiment possessed ability to form an organ of all parts and normal to the degree of carrying on circulation.

DISCUSSION

The results of the experiments bear chiefly upon the following questions: Has the heart rudiment the power of self-differentiation? Is the rudiment equipotential? Is polarity determined within the rudiment or is it influenced by the organic environment?

As stated in the introduction, the heart was first called a self-differentiating system by Ekman ('21), who found by explantation experiments in Bombinator that the heart would develop outside the body. Stöhr also considers that the heart is self-differentiating to a certain extent, but not to so great an extent as Ekman does. There seem to be differences of opinion concerning the importance of neighboring tissues for normal development, and also differences in emphasis on the various factors for development of the form of the heart.

Results of the heterotopic experiments given in the present paper show that the presence of neighboring tissue is not necessary for the early development of the heart. In most of the experiments there was no evidence of any part of liver, gut, etc., with the transplanted heart. The organ developed in a pericardial cavity with pericardium surrounded only by ectoderm and the yolk-containing cells. Pulsations of normal rate occurred and differentiation into sinus, atrium, ventricle, and bulbus took place, indicating, therefore, considerable power of self-differentiation in the absence of neighboring organs. The transplanted hearts were smaller than normal and were sometimes atypical, having the sinus and bulbus especially small and poorly differentiated. In considering the atypical conditions, the following facts should not be overlooked. First, the heart rudiment is of a very plastic nature, as indicated by pathological studies and as has been shown in experimental work, and accordingly the effect of operations might be expected to be greater than in some other organs. Secondly, in heterotopic transplantations, the heart being in an abnormal position, it is unable to become normally connected with the aortic arches and vitelline veins and ducts of Cuvier. This necessarily brings about an atypical condition and also probably influences the extent of development of sinus and bulbus, especially when these parts become connected to no vessels whatever.

In later development factors other than self-differentiation play an important part. In experiments in which there was absence of circulation through the heart, development progressed normally for a few days only; later there was slowing of the pulse rate and atrophy of the organ, due to lack of blood supply and the absence of complete function. Since the development of the structure of an organ is correlated with its functioning, especially in later stages, the absence of the blood stream affected development not only because of absence of blood supply, but also because of absence of complete function. The liver may also have an effect, since, according to Asher ('23), it has a chemical regulatory influence on the heart beat and therefore on the function. The flowing blood stream is also of importance in later development as a factor influencing the development of form. This has been discussed by Greil ('03), Beneke ('20), Benninghoff ('23), and more recently by Stöhr.

With respect to the curvature of the heart, self-differentiation plays a relatively unimportant part. According to Spemann ('06), Pressler ('11), Meyer ('13), Stöhr ('24 b), and Ekman ('24 b), the heart possesses within itself a tendency for curving. The particular type of curvature is, however, as also stated by Stöhr and Ekman, influenced by outside factors. That the type of curvature is not determined from within is seen from the heterotopic experiments of the present paper, since the curvature was often variable and atypical. In the rotation experiments the type of curvature resembled the normal rather than the rotated condition, also indicating that the type of curvature was not determined from within. The form of the individual parts after rotation was retained to a considerable extent, that is, the sinus when placed anteriorly developed more like a sinus in form than like the bulbus, but the parts were so arranged in relation to each other as to give the organ as a whole an approximately normal type of form and curvature.

The conclusions concerning self-differentiation may be summed up as follows: the rudiment is self-differentiating in that it will develop a pulsating organ normally differentiated into all parts to a degree of development corresponding to the stage when circulation normally begins, in that by factors from within which bring about differential growth a beginning of form is produced for the individual parts, and in that there exists within the rudiment itself a tendency for curving. The general form and type of curvature are strongly influenced by the contour of the surrounding tissue. In later development the blood stream and perhaps other factors are important for continued growth and differentiation and for increasing and modifying the general curvature already established by the direct effect of the organic environment and for molding the structure and form of the individual parts.

Is the heart an harmonic equipotential system? Ekman ('21) by dividing the rudiment in the midline, was able to produce two hearts from one rudiment in Bombinator. More recently, Ekman ('24 b) has described the development of as many as three hearts from one embryonic rudiment. Stöhr has also found that two hearts will develop from one rudiment, and in the experiments of the present paper it has been shown that two hearts functional to the degree of having circulation can be made to develop from one rudiment. On the other hand, in explantation and in heterotopic transplantation experiments Stöhr has found that unequal differentiation frequently occurs and that sometimes only two or three parts rather than the normal four were differentiated. In the heterotopic transplants described in section 9 whenever differentiation to any extent occurred in any part, all parts of the heart were present, although the sinus and bulbus were sometimes relatively small and atypical, and doubtless a factor in the atypical development of these parts was the fact that there was absence of normal connection with vessels anteriorly and posteriorly.

In heterotopic transplantation experiments there was no development of vessels from the anterior or posterior parts of the transplanted hearts; connections with the vascular system when present were connections with small vessels developing in situ in the host. After extirpation experiments, aortic arches, the vitelline veins, and ducts of Cuvier developed in situ. In connection with the aortic arches a somewhat typical truncus arteriosus often developed, and in connection with the veins a small sinus-like chamber was frequently formed. Apparently totipotentiality does not extend into the truncus arteriosus nor into the posterior part of the sinus; at least, these parts do not possess the power to differentiate into a small heart of all parts.

The experiments testing equipotentiality were similar to experiments of Harrison ('18) on the fore-limb bud, and the results were very similar except that the number with atypical development was somewhat larger. The normal development obtained after such experiments, namely, after experiments of extirpation of right, left, or anterior halves; after transplantations giving two right, two left, two anterior or two posterior halves, and after superimposing rudiments, as well as the functional double hearts developed after the rudiment had been split in the midline, show the heart rudiment to be an equipotential system at the standard operating stage.

The differences concerning equipotentiality as given here for Amblystoma and as given by Stöhr ('24 d), working mostly with Bombinator, may be due to use of different stages for operating. Stöhr states that most of the operations were made at the tail-bud stage of development. The heart of Bombinator at that stage, according to figures given by Ekman ('24 b), corresponds in development to the heart of Amblystoma of about stage 30, which is later than the standard operating stages used in the present experiments. In Amblystoma equipotentiality practically disappears between stages 29 or 30 and stage 33, as shown in section 15 of the present paper, and if a similar course is followed in Bombinator equipotentiality would be expected to disappear at about the tail-bud stage. Moreover, there is reason for believing that in Amblystoma the polarity of the anteroposterior axis of the heart rudiment is determined before that of the transverse. Accordingly, if a similar course is followed in Bombinator, stages might be expected to exist at which operations of splitting the rudiment into right and left halves would be followed by development of two hearts, as found by Ekman and also by Stöhr, and yet at these same stages there might occur unequal differentiation and failure to develop all parts of the heart from fragments not containing tissue from the whole length of the long axis, as found by Stöhr in explanted fragments and in heterotopic transplants.

The problem of axial determination in the dorsoventral direction does not readily lend itself to experimentation. With respect to the mediolateral direction, which, owing to the shifting of the rudiment, corresponds to the dorsoventral direction in early stages, the problem can be investigated, but not as clearly as in the limb, for the organ develops at a median position and it is impossible at the standard stage to transplant the rudiment as a whole in such a way as to change it from the right side to left. Transplantations of halves at the stage when the rudiment is median have been made, and these change the mediolateral axis of the half. Combinations of halves in this way might be expected frequently to give reduplications; such was not the case, and apparently the determination in this direction, if present, is not irreversible at the stages operated upon.

The postero-anterior direction was found to be irreversible at all stages of operation, being similar in this respect to the fore-limb bud as described by Harrison ('21). Rotations of 180° about the dorsoventral axis caused the heart to develop with its parts arranged in sequence opposite to normal. According to Schübel and Stöhr ('24), rotation at open medullary-fold stage does not produce reversal in development. According to figures given by Ekman ('21) for Bombinator, the heart of Bombinator at the open medullary-fold stage is at a stage of development corresponding to the tail-bud stage of Amblystoma. In Amblystoma rotation produced reversal at stages earlier than the tail-bud stage, stage 25; as early as stage 22, when the mesoderm fold ends far laterally, bilateral rotation experiments produced rotated hearts. Accordingly, Bombinator and Amblystoma seem to differ with respect to the stage of development of the heart rudiment when determination of the postero-anterior axis occurs. The results show that in Amblystoma determination of the postero-anterior axis takes place at stages considerably earlier than the stage when equipotentiality is lost. Since the potency to form a heart extends in the neighboring mesoderm farther posteriorly than anteriorly with respect to tissue normally forming the heart, and since in regeneration experiments, also, development from the posterior part is greater than from the anterior part, the gradient along the axis is probably postero-anterior rather than anteroposterior. This agrees with the fact that the sinus is physiologically dominant.

Situs inversus cordis was described by Spemann ('06) in amphibian embryos having situs inversus viscerum. Similar results were later produced by Pressler ('11) and Meyer ('13), and more recently Spemann and Falkenberg ('19) have found that in Triton embryos with fore part double the right half often showed situs inversus viscerum and cordis. Mangold ('21) has also found situs inversus in the right twin in Triton. The results of experiments given in section 12 of the present paper also show that situs inversus cordis occurs on the right side, and in this respect the results are similar to results described by Ekman ('24 b) for double hearts. When the rudiment was split into two parts and two hearts developed, the left one was normal, the right always showed situs inversus, being the mirror image of the left.

From a study of normal heart rates in which it was found that the rate of beat differed for the various stages, but was approximately the same for all embryos at similar stage of development when at constant temperature, it might be expected that an operated embryo having approximately normal rate would have a normal heart. It was found, however, that the rate being normal did not indicate a heart of normal size, but rather one of normal differentiation. In heterotopic transplants the hearts were smaller than normal, but usually pulsated at normal rate, except when the heart was extremely small. The correlation between rate of beat and development of the heart was not a correlation between rate and size, but between rate and stage of development.

SUMMARY

1. When the mesoderm normally forming the heart is extirpated at stages earlier than stage 29, surrounding mesoderm possesses the potency to develop a normal heart.

2. By extirpation of a larger area and covering of wound with ectoderm and mesoderm from the flank, development of the heart is prevented.

3. Bilateral extirpation at stages when the mesoderm ends relatively far up on the side of the body prevents development of the heart. The absence of endocardium after bilateral extirpations indicates that the endocardium has its origin from the splanchnic mesoderm in the urodeles as in other forms.

4. Embryos without circulation usually live from two to three weeks, sometimes longer, but differentiate normally for only the first few days following the operation.

5. Orthotopic transplantations at standard stage (stages 25 to 29, figs. 1 to 6) give normal hearts with circulation when the transplant is made with antero-anterior orientation. After anteroposterior orientation, reversed hearts develop with pulsations from anterior to posterior in direction and without circulation.

6. Orthotopic, bilateral transplantations with anteroposterior orientation at early stage (preceding tail-bud stage) produce hearts which are usually double and always reversed.

7. Heterotopic transplants develop in accordance with the original orientation of the transplant. Their development indicates that the heart in its early stages of development is

self-differentiating, except for type of curvature. The latter is largely determined in the early stages by the contour of the cavity in which the heart develops.

8. In heterotopic transplants small hearts, except when extremely small, pulsate at normal rate. The pulse rate was found to be correlated with degree of development, but not with size of the heart.

9. Superimposed rudiments develop functional hearts with circulation when the transplants are made with antero-anterior orientation, but when made with anteroposterior orientation the direction of beat is atypical and circulation absent.

10. By removing a median area and thus splitting the rudiment into two parts, two hearts may be produced functional to the extent of having circulation. In form the left approximates the normal; the right invariably shows situs inversus.

11. After removal of an anterior half or a right half, at the standard operating stage, the remaining half develops a heart of all parts.

12. After extirpation of parts just preceding beginning of pulsations, a complete heart often developed. The process of development, however, was one of regeneration. After extirpations of parts at stages with beginning pulsations, regeneration does not occur.

13. After transplantation of halves giving combinations of two anterior or two posterior halves with normal orientation, normal hearts develop which are functional to the degree of having circulation in 50 per cent of the positive experiments. Likewise, combinations of two right or two left halves give normal hearts with circulation in 50 per cent of the positive experiments.

14. The results of the experiments indicate that the heart of Amblystoma punctatum is an equipotential system in the sense that, *a*) any part as large as a half possesses potency for developing a whole of all parts; *b*) that one rudiment is capable of forming two functional organs, and, *c*) that two rudiments can be made to form one functional organ.

15. At the earliest stages operated upon, stages considerably preceding the loss of equipotentiality, there exists within the rudiment itself determination of the postero-anterior axis.

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THE RELATIONSHIP OF RHYTHMS TO NUTRITION AND EXCRETION IN PARAMECIUM[1]

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FIFTEEN CHARTS

CONTENTS

Introduction 374
Materials and methods 375
Observations 381
  A. Paramecium aurelia 381
    Culture I 381
    Culture II 383
    Combination of results from cultures I and II 384
    Culture III 385
    The number and size of food vacuoles in Paramecium aurelia 386
    Rates of the anterior and posterior contractile vacuoles 389
  B. Paramecium calkinsi 390
    Culture I 390
    Culture II 392
    Combination of results from cultures I and II 394
    Culture III 394
    The number and size of food vacuoles in Paramecium calkinsi 396
    Rates of the anterior and posterior contractile vacuoles 397
  C. Comparison of Paramecium aurelia and Paramecium calkinsi reared synchronously 399
  D. Paramecium caudatum 399
    Culture I 399
    The number and size of food vacuoles in Paramecium caudatum 401
    Rates of the anterior and posterior contractile vacuoles 402
Discussion 404
Summary 410
Literature cited 412

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INTRODUCTION

Early in the study of pedigree races of Protozoa, major fluctuations in their reproductive activity were noticed. Calkins ('02) used the word 'cycle' to describe the phenomenon whereby vitality started at a high point after conjugation and gradually fell off as senescence came on, and the race finally perished unless rejuvenescence occurred. Later studies by Woodruff ('05) demonstrated the presence of minor fluctuations in the reproductive rate; these minor fluctuations were called 'rhythms,' and a rhythm was defined as "a minor periodic rise and fall of the fission rate, due to some unknown factor in cell metabolism from which recovery is autonomous." A cycle was made up of a number of rhythms; both terms were, of course, applied to fluctuations in division rate only.

These rhythms have been noticed in all species of protozoa studied by the pedigree method, and the evidence is especially striking on Paramecium aurelia, where observations have been most extensive. Employment of a constant culture medium has not served to eliminate rhythms, although it has eliminated the cycles (Woodruff and Baitsell, '11 a). Nor is temperature a crucial factor in these rhythmical changes (Woodruff and Baitsell, '11 b).

Gradually the idea became crystallized that these rhythms were due to intrinsic rather than to extrinsic causes. This view was strengthened by the discovery of endomixis in Paramecium aurelia (Woodruff and Erdmann, '14), Paramecium caudatum (Erdmann and Woodruff, '16), and Paramecium polycaryum (Woodruff and Spencer, '23) coincident with rhythms. It was thought that the secret of rhythms had been solved, because endomixis always occurred at the lowest point of the curve for reproductive activity. In other words, it was assumed that rhythms were essentially the visible physiological demonstration of the hidden phenomenon of endomixis.

However, Woodruff ('21) and Spencer ('24) have shown that endomixis does not occur in Paramecium calkinsi, but in spite of that fact rhythms are present. Moore ('24), working on Spathidium spathula found that endomixis never occurred except during encystment, and as long as encystment was prevented, endomixis did not occur—and yet rhythms were present as usual. Results of these investigations show that endomixis is not necessarily an accompanying phenomenon to rhythms. The sum of knowledge thus far amassed on the problem of rhythms reverts to the statement of Woodruff made twenty years ago—rhythms are "due to some unknown factor in cell metabolism."

The present work was undertaken in an attempt to throw additional light upon rhythms and to determine the relation between these fluctuations in the division rate and the variation of metabolic activities as indicated by the rate of pulsation of the contractile vacuoles and the size and number of food vacuoles formed—in brief, to determine, if possible, the factors underlying rhythms.

The author takes great pleasure in acknowledging his indebtedness to Prof. Lorande Loss Woodruff, at whose suggestion this investigation was begun, for his valuable advice and constructive criticism throughout the course of this study, and also to Prof. J. W. Buchanan for many helpful comments.

MATERIALS AND METHODS

The Infusoria which formed the basis of this experimental work were races of Paramecium aurelia, Paramecium calkinsi, and Paramecium caudatum. The first two species were selected purposely, because it was an established fact that endomixis occurred in the former and not in the latter. In addition to these two, observations were conducted on Paramecium caudatum, where endomixis was also known. Each species was bred in pedigree cultures.

All individuals of Paramecium aurelia were derived from one animal from Woodruff's pedigree line on June 23, 1924, then in approximately the 15,000th generation.

The cultures of Paramecium calkinsi studied were all descended from one individual from a race collected during August, 1924, at Woods Hole, Massachusetts.

The Paramecium caudatum were progeny of an individual isolated on December 20, 1924, from a culture jar in the Osborn Zoölogical Laboratory. This material had previously been collected from a fresh-water pool in the vicinity of New Haven.

The pedigree cultures were established as follows: With the eight individuals present after three divisions of the original animal of any one species in question, two cultures of four lines each were started. The two cultures were designated I and II, and the separate lines of each were numbered from 1 to 4. When an individual in line 1 had divided once, an animal was used to continue line 1, and the sister cell was put on another slide and designated 'supply.' Thereafter, the offspring of the individual in line 1 were used to continue both line 1 and the supply line, while the animals in the supply line were used in the daily experiments. In other words, the animals observed were always 'first cousins' of the particular animal in the main line for that certain day. In the event that the division rates of the main and supply lines were not identical, no observations were made, so the records of division rate and daily observations on individuals correspond in all instances. If, for example, the main-line animal had divided once since the last isolation and the supply animal had not divided, no observations were made on this supply animal, because the division-rate record for that day would have indicated one division, whereas no such multiplication had occurred in the supply line. Cases in which the main line and the supply lines divided at different rates were remarkably infrequent.

Every day throughout the course of observations, one animal from each line was examined, i.e., four Paramecia from each culture. In the event of a discrepancy between the division rates of main and supply lines, two animals from one of the other lines of the culture under consideration were examined; in that way, the averages were maintained constant, i.e., four animals were examined from each culture every day.

All of the protozoa were reared on depression slides in moist chambers at room temperature. The media were changed daily on all animals, and cross infection served to keep the bacterial flora constant in all lines of each species under observation. In all species, each animal was given two drops of culture medium on a slide, and, therefore, each animal was furnished daily with the same volume of the given culture medium for that particular species.

At the inception of cultivation of the animals, it was found that all three species could not be bred equally satisfactorily on the same type of culture fluid. Accordingly, the standard was set for each species by experimentation, and then the most satisfactory medium was kept constant for a given species throughout the course of experiments.

In the case of Paramecium aurelia, one drop of standard beef extract and one drop of 1 per cent hay infusion constituted the daily nourishment of each isolated individual. The beef extract was a 0.025 per cent solution of Liebig's beef extract, in glass-distilled water. Fresh hay infusions were prepared daily by boiling 5 grams of timothy hay in 500 cc. of tap-water for five minutes; this infusion was used twenty-four hours after preparation.

It was found that the race of Paramecium calkinsi which was employed would not thrive unless the medium was slightly saline. No doubt, this was correlated with the origin of this strain from Woods Hole, where many of the pools have a relatively high salt content. Accordingly, P. calkinsi was cultured in two drops of a medium consisting of 1 part of 1 per cent salt solution (C.P. in glass-distilled water) and 4 parts of standard hay infusion.

Paramecium caudatum always received two drops of standard beef extract.

Although each species was raised in a medium slightly different from the other two, there was a close relationship between the several culture media, because the hay infusion contributing to the welfare of P. aurelia and P. calkinsi for a given day came from the same flask of this preparation;

the beef extract which formed part of the culture medium of P. aurelia and all that of P. caudatum for a given day came from the same tube of extract.

The external factor of barometric pressure has not been considered, since Woodruff and Baitsell ('11 c) have shown that this factor produces no effect on colorless Paramecia. These authors also were of the opinion that daylight does not influence the division rate of these animals. Recent work by Packard ('25) indicates that light does have an influence on the permeability of the organism. However, that factor need not be considered in the experiment, since all of the cultures were located near a north window and all cultures directly compared were carried synchronously, and, therefore, were similarly subjected to such changes in illumination as did occur.

All cultures were carried at room temperature and were, therefore, subject to minor variations. A complete record of thermal conditions was made possible by the use of a recording thermometer. However, that factor need not be considered in the experiment, since all cultures directly compared were carried synchronously, and, therefore, were similarly subjected to such changes in temperature as did occur.

Observations on the contractile vacuoles and the food vacuoles had to be made under conditions which rendered the Paramecium sufficiently quiet to be critically studied and yet not abnormal in shape nor in an abnormal environment. With the purposes of the experiment in mind, it was at once obvious that no chemical inhibitors, such as propyl alcohol, could be used; neither was the use of the hanging-drop method, cotton fibers, nor quince-seed jelly satisfactory. With only two or four animals available in each line (depending on whether the supply line had divided once or twice), it was imperative that a very precise technique be devised in order to make observations and yet not lose the few animals at the disposal of the experimenter. Accordingly, the following method was devised: Two pieces of fine glass wool about

$\frac{1}{4}$ inch long were placed on a clean glass slide about $\frac{1}{8}$ inch apart. Between these two fragments of glass wool, an animal was introduced in a small drop of culture fluid. A fragment of cover-slip was carefully lowered upon the drop containing the animal. It was found that ordinary circular cover-slips were too large for the purpose; pieces of cover-glass about $\frac{3}{8}$ inch square were best. The strands of glass wool supported the cover-slip and prevented it from smashing the Paramecium in the thin chamber thus formed between slide and cover. Glass wool, 18 μ in diameter, was selected, because it was of the proper thickness to form a suitable chamber to accommodate the animals under observation. Due to its uniform thickness and non-toxicity, glass wool is the ideal material for this work.

Up to this point the preparation was made on the stage of a binocular microscope; the slide was then transferred to the mechanical stage of a compound microscope, where all subsequent observations were made.

Usually the Paramecia were held practically stationary, with possibly minute swayings of the cell which did not interfere with the initial steps in observation. Quite often the animal would be allowed to swim about within the limits of the field while the intervals between contractions of the pulsating vacuoles were recorded. In order to stop the movements of the animals altogether, it was only necessary to withdraw a tiny amount of water by means of filter paper. The food vacuoles could then be easily counted and measured.

Daily observations were always made between the hours of 7 A.M. and 11 A.M. Contractile vacuole pulsations were observed at a magnification of 100 diameters and the rate determined by means of a stop-watch. One or two contractions of the anterior vacuole, let us say, were noted, and then a similar number of contractions of the posterior vacuole were timed. By this method, the two vacuoles were observed in the same state of fatigue; in other words, if one had been observed for the customary number of contractions, it is conceivable that the other vacuole might have suffered some physiological modification, e.g., from excess of CO_2 in a small amount of fluid; but this alternation of observations rules out such an objection. In every animal five contractions of each vacuole were noted. It was not practicable to make more than that number of observations on vacuole contractions because of the possibility of an effect from the abnormally restricted environment of the animal before other data could have been gathered. Since one animal from each of four lines was observed, there was a total of twenty intervals between contractions measured daily. The averages of these twenty periods between contractions in seconds were in turn averaged, and from this figure the contractions per minute were computed. These values were, then, averaged at five-day intervals, and the resulting figure was used to plot the variations in graphic form.

Food vacuoles were counted under a magnification of 430 diameters; the number of food vacuoles was taken in all lines and averaged for each culture for every five days; values obtained in this way were plotted.

The diameters of food vacuoles were measured by means of an ocular micrometer disc and such a combination of lenses (at a magnification of 570 times) that one division of the micrometer scale equalled 2 μ. All measurements are therefore expressed in multiples of 2 μ. Since food vacuoles are spherical in shape, the diameters will serve as satisfactory units for expressing the sizes. Results of all food-vacuole measurements in the three species of Paramecium observed are plotted in figures 6, 12, and 15.

The division rate was also averaged for all four lines of each culture for every five days.

In the preparation of all animals for cytological study, Schaudinn's fluid was used as the fixing agent. Animals were stained in Ranvier's picrocarmine or in Delafield's haematoxylin. Either stain proved equally satisfactory for the purpose in hand.

OBSERVATIONS

A. Paramecium aurelia

Culture I. Culture I was started on June 28, 1924, from an animal of Woodruff's pedigree line, then in approximately the 15,000th generation, and bred for eighty days, during which time ninety-four generations were attained. The record of the various phenomena observed in culture I are shown in figure 1.

By a study of this figure it will be seen that low points on the graph for reproductive rate coincide with the appearance of endomixis, at 15, 25, 50, and 75 days after beginning the observations. The division rate data for the entire eighty days of the experiment shows that the lowest rate of division during any five-day period was 0.3 divisions per individual and the highest was 1.8.

Depressions in the rate of contraction of the excretory vacuoles is also synchronous with the occurrence of endomixis; in fact, the relations of the fluctuations of the rate of pulsations of contractile vacuoles and of division rate are so definite that endomixis can be predicted by examination of either graph.

It is noticeable that the rates of the anterior and posterior contractile vacuoles, when contrasted, vary considerably; i.e., during one five-day period the anterior vacuole may beat more rapidly than the posterior and again the reverse may be true. In nine of the five-day periods, the posterior vacuole is ahead of the anterior; in three cases they are identical, and in the remaining four, the anterior vacuole pulsated more rapidly than the posterior. A more detailed consideration of this variation between the anterior and posterior contractile vacuoles is taken up below, after all cultures for this species have been described.

The fluctuations in the average number of food vacuoles formed per individual is one of continuous ascendency; i.e., each graph representing the number is higher than the preceding one, although depression points do agree with endomixis. This continuous rise has been interpreted as an external expression of acclimation to environment, with special reference to a constant food supply. The smallest average number of food vacuoles per individual over any five-day period was found to be 7.1, while the highest average number was 15.1.

These closely related results indicate that all are due to some common intrinsic factor of the animals, since the external factors were practically constant with the exception of the temperature, and this was the same for all cultures compared.

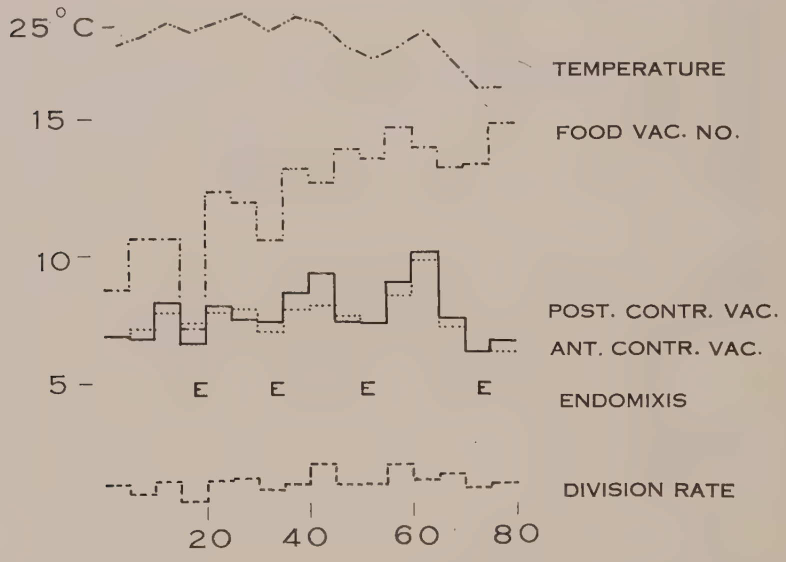

Fig. 1 Record of observations on culture I of Paramecium aurelia for eighty days plotted at five-day intervals. The ordinates from *0* to *15* have the same numerical value; the abscissas represent five-day periods. The temperature record is inserted at the top of the figure. Endomictic periods are designated by the letter *E*.

A critical study of all the data indicates that at times a lowering of temperature corresponds to a lowering of the various phenomena under observation, and at other times a rising temperature is accompanied by depressions in the same phenomena. Accordingly, it is clear that, although, as is well known, temperature markedly affects the various metabolic activities of the organisms, it is not the crucial factor underlying rhythms. This is in agreement with the conclusions of Woodruff and Baitsell ('11 c) and Spencer ('24).

Culture II. Culture II originated from the same animal which constituted the source of culture I. Observations on culture II were concluded at the 101st generation after it had lived synchronously with culture I for eighty days.

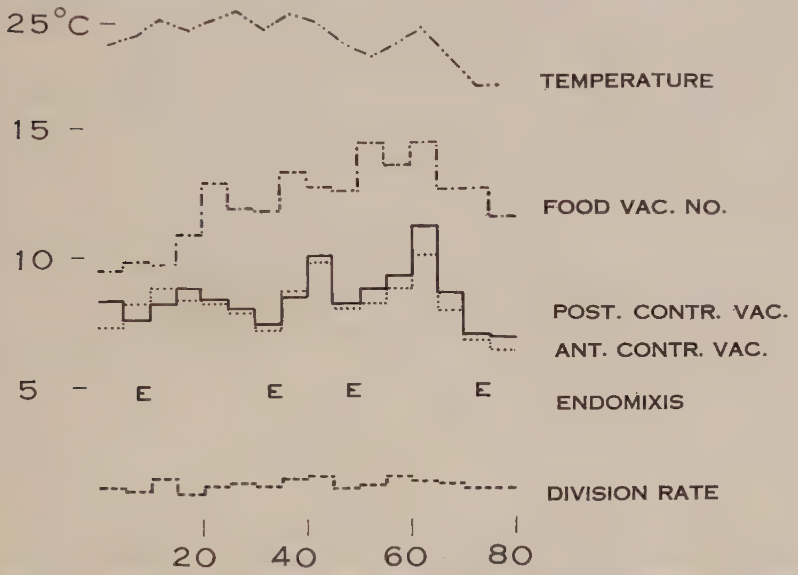

Fig. 2 Record of observations on culture II of Paramecium aurelia for eighty days plotted at five-day intervals. The ordinates from *0* to *15* have the same numerical value; the abscissas represent five-day periods. The temperature record is shown at the top of the figure. Endomictic periods are designated by the letter *E*.

Figure 2 is a record of behavior of culture II. Here again the low points in fluctuations of metabolic activity and the low points of the rhythms in the division rate coincided with endomixis; in fact, endomixis could be predicted with certainty by examination of contractile vacuole graphs. In the division rate there is less variation than in culture I, since here the low point in reproductive activity is at 0.8 division per individual, while the high point is 1.5.

In this culture, too, the comparative rates of anterior and posterior pulsating vacuoles are subject to variation, although on the average the posterior vacuole beats more rapidly. In thirteen cases the posterior vacuole pulsated more rapidly, while in only three cases did the anterior vacuole show a higher rate of contraction. The highest average of the anterior contractile vacuole was at 10.0 contractions per minute, while the lowest rate was 6.3 contractions per minute. Examination of five-day averages discloses the fact that the posterior vacuole never pulsated more slowly than 6.8 times per minute nor faster than 11.2 times per minute.

The curve for food vacuoles shows the characteristic ascending rhythm noted in culture I, except that in this culture a falling off is noticeable at the end of the experiment concurrent with a marked drop in excretory activity.

The first five-day average shows but 9.5 food vacuoles per individual; at the high point, forty-five days later, 14.4 gastric vacuoles per individual were noted.

The temperature record is identical with that of culture I, since both cultures were maintained at the same time and place under identical thermal conditions.

Combination of results from cultures I and II. A survey of all data from cultures I and II is afforded by a combination of averages of data presented in figure 3. This treatment of the data is legitimate, since, for all practical purposes, cultures I and II may be regarded as one culture comprising eight lines.

In the curves for division rate, the pulsations of anterior and posterior contractile vacuoles and the number of food vacuoles formed—all show depression points synchronous with endomixis.

The last three endomictic periods happened in both cultures at the same time. The first example of endomixis occurred about nineteen days apart. Manifestly, in this one instance, the average of the two cultures will tend to shift the point of depression and make it appear halfway between the actual occurrences of endomixis.

Culture III. Another culture of Paramecium aurelia was bred from January 5 to February 8, 1925; in that time it attained fifty-five generations. The initial animal to start culture III was taken from a mass culture in a flask seeded from an individual Paramecium aurelia at the close of culture II. Therefore, culture III came from the same strain of animals as those used in cultures I and II. The results of carrying and examining this culture are shown in figure 4.

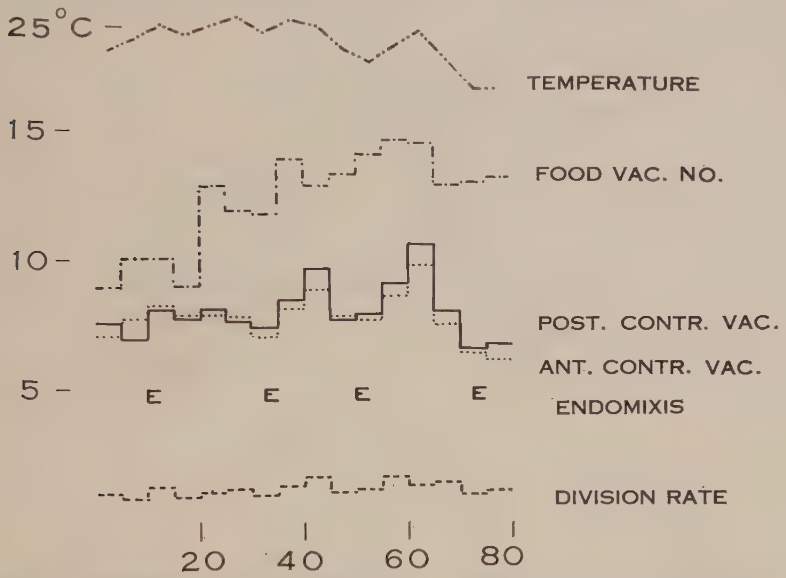

Fig. 3 Combined records of observations on cultures I and II of Paramecium aurelia for eighty days plotted at five-day intervals. The ordinates from *0* to *15* have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure. Endomictic periods are designated by the letter *E*.

During the thirty-five-day period of this experiment endomixis was demonstrated twice, and it occurred at the low points in metabolic activity. Due to some unknown cause, the posterior contractile vacuole rate failed to drop in accord with the anterior vacuole as it did in all other instances. In this connection it is interesting to note that the first slight drop in temperature is coincident with the greatest number of food vacuoles; but later a fall in temperature succeeded by a minor rise was accompanied by a decline in the number of food vacuoles formed.

From a study of these results alone (cultures I, II, III) it would appear that the fluctuations in the division rate and in contractile and food vacuole phenomena in Paramecium aurelia are the direct results of endomixis. However, the data for other species to be presented beyond make this conclusion untenable.

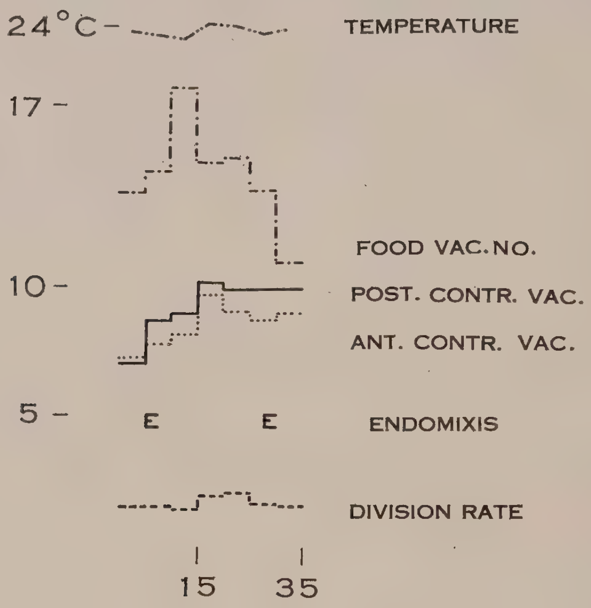

Fig. 4 Record of observations on culture III of Paramecium aurelia for thirty-five days plotted at five-day intervals. The ordinates from *0* to *17* have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure. Endomictic periods are designated by the letter *E*.

The number and size of food vacuoles in Paramecium aurelia. Various data collected from all cultures of Paramecium aurelia will now be presented as representative of this particular race, rather than of a particular culture, since the individual cultures afford no significant differences. During the history of the cultures, 719 animals were examined, with a grand total of 9105 food vacuoles. The number of vacuoles found in a Paramecium varied from 0 to 28. A graph of the distribution of the number of vacuoles per individual is shown in figure 5: 89 animals contained 12 vacuoles per individual; 77 had 13 vacuoles per individual; 74 Paramecia had 14 vacuoles each. Thus 240 individuals, or 33 per cent of the number of animals examined, formed from 12 to 14 vacuoles each.

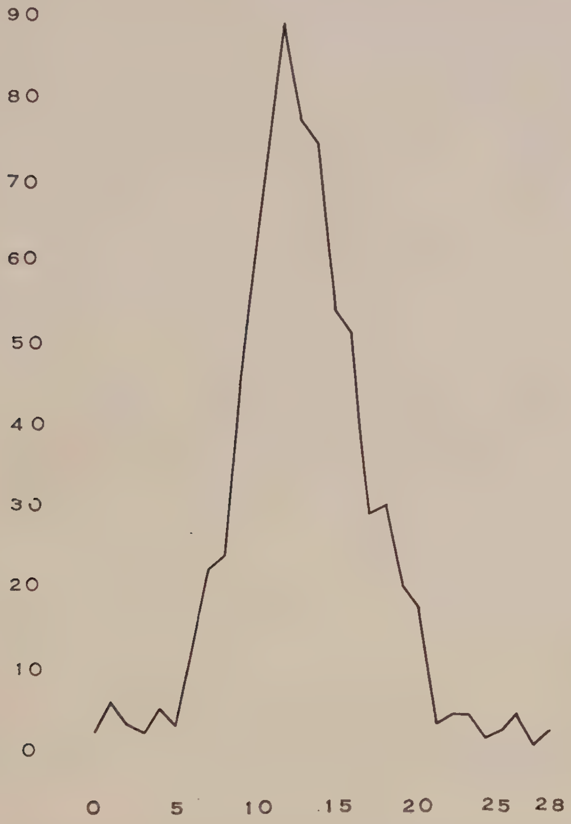

Fig. 5 This graph shows the distribution of the number of food vacuoles per individual in Paramecium aurelia. The ordinates represent the number of individuals and the abscissas represent the number of vacuoles.

The diameters of the 9105 food vacuoles were also carefully measured. Figure 6 gives a curve of the distribution of the vacuoles with reference to their diameters. It is evident that the smallest vacuole seen was 4 μ in diameter, while the largest was 30 μ in diameter. There were 3607 vacuoles with a diameter of 10 μ; 2515 vacuoles were 12 μ in diameter, and 1241 were 14 μ in diameter. Therefore, 7363 (or 80.8 per cent) of the 9105 vacuoles measured were from 10 to 14 μ in diameter.

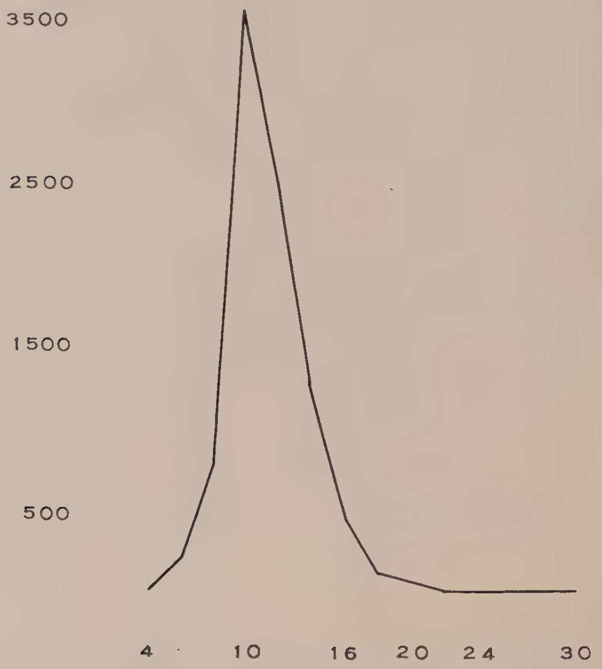

Fig. 6 This graph shows the distribution of 9105 food vacuoles in Paramecium aurelia, with reference to their diameters. The ordinates represent the number of vacuoles and the abscissas represent the diameters of the vacuoles in micra.

These results on the size of gastric vacuoles corroborate previous results obtained from mass cultures of Paramecium aurelia.[2] In that study 11,815 food vacuoles were measured. The size of these vacuoles was within the limits of 4 and 28 μ, and the majority were 10 to 14 μ in diameter; 9063 vacuoles were either 10, 12, or 14 μ in diameter.

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[2] Digestion in Protozoa: Thesis deposited in the Library of Lafayette College, Easton, Pennsylvania, by W. Byers Unger.

Thus from a total of 20,920 food vacuoles measured in Paramecium aurelia, the data show that the average size of the individual food vacuoles formed is from 10 to 14 μ in diameter.

Rates of the anterior and posterior contractile vacuoles. It has been shown that, on the average, the posterior contractile vacuole of Paramecium aurelia pulsates more rapidly than the anterior. A survey of all five-day periods in all three cultures shows that the anterior vacuole averaged 7.99 contractions per minute and the posterior vacuole contracted 8.37 times per minute. In view of the possibility that these figures do not represent a significant difference—i.e., they are within the limit of probable error—a calculation was made on the basis of the original observations in seconds. This revealed a mean of 7.80 seconds between contractions of the anterior vacuole and a mean of 7.26 seconds between pulsations of the posterior vacuole. Expressed in terms of contractions per minute, this is equivalent to 7.69 contractions for the anterior and 8.26 contractions for the posterior vacuole. The difference in values obtained by the two methods (on the basis of seconds and on the basis of daily averages expressed in contractions per minute and then averaged for five-day periods) is due to the less exact method used in computing five-day averages whereby decimals beyond tenths were disregarded.

TABLE 1

Comparison of contractile vacuole rates in Paramecium aurelia

	NUMBER OF CONTRACTIONS	RANGE IN SECONDS	MEAN	STANDARD DEVIATION
Anterior vacuole	3435	5 to 23	7.80±.02	1.75±.01
Posterior vacuole...........	3300	4 to 22	7.26±.02	1.86±.01

Probable difference, 0.028
Observed difference, 0.420

From the analysis of the data gathered by the more exact method, the probable difference between contractions of the anterior and posterior vacuoles was found to be 0.028 second, while the observed difference was 0.420 second, thus indicating conclusively that the posterior vacuole in Paramecium aurelia actually does, on the average, pulsate more rapidly than the anterior vacuole.

B. Paramecium calkinsi

Culture I. One Paramecium calkinsi was isolated on September 20, 1924, from a culture that had been collected at Woods Hole, Massachusetts, in the summer of 1924; this animal formed the foundation of all the individuals studied in this species. On October 4, 1924, observations were begun, and they continued for eighty days until December 23, 1924. During that time the culture attained eighty-four generations. Figure 7 shows a record of the various phenomena observed in culture I.

Twice during the history of the culture, the division rate fell to 0.5 division per individual per day, and the highest point reached was 2.0 divisions per individual per day.

Endomixis has never been demonstrated in this species by previous workers. Throughout the course of this particular investigation at least four sister cells were examined cytologically every day, but the results revealed no nuclear change comparable to endomixis. Thus the result of previous workers (Woodruff, '21, and Spencer, '24) was corroborated on this point.

It was noticed that on certain days the micronuclei showed up distinctly and on other days they were either indistinct or altogether invisible. When one bears in mind the very small size of the micronuclei in this species, it is probable that slight variations in staining or their position below the macronucleus rendered them invisible. On the assumption that this diversity in the appearance and disappearance of micronuclei might be related to rhythms in some way, all the material was studied cytologically under oil-immersion lens. But no relation could be found between demonstrable micronuclei and rhythms, and accordingly the data at hand indicate that it is without significance for the problem under consideration.

Depressions in the rate of the contractile vacuoles are, in general, synchronous with low points of rhythms in the division rate. Although fluctuations of the contractile vacuole rates of P. calkinsi are present, they are less pronounced than those of P. aurelia; pulsation rates extend only over the short range between the extremes of 3.8 and 4.4 contractions per minute. It is also noticeable that the rates of the two vacuoles are never very much at variance; in fact, the rates of the two are often identical, and at other times the alterna-

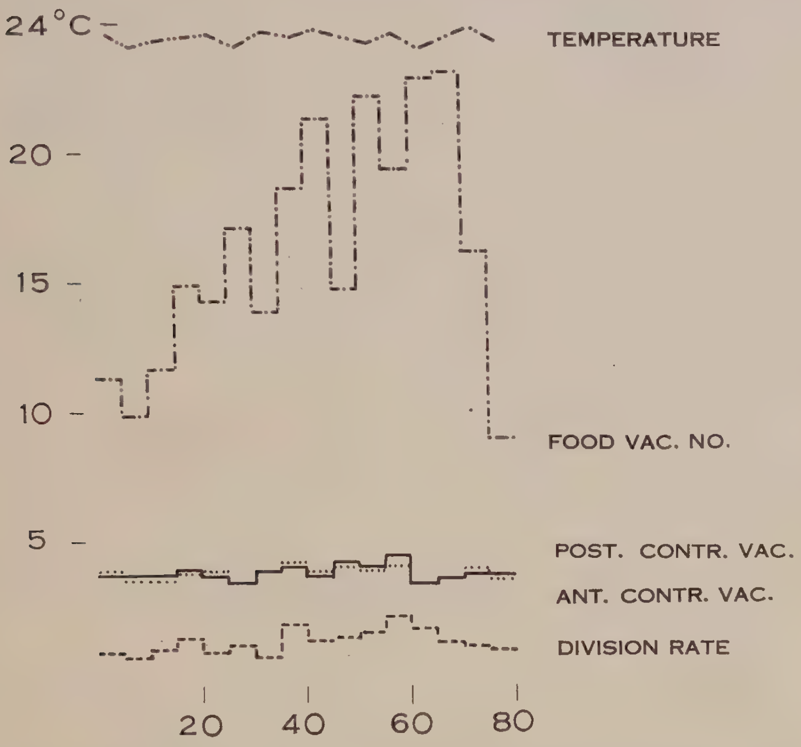

Fig. 7 Record of observations on culture I of Paramecium calkinsi for eighty days plotted at five-day intervals. The ordinates have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure.

tions on successive days counterbalance one another. A more detailed consideration of this fluctuation between the rates of the anterior and posterior contractile vacuoles is taken up below, after all cultures of this species have been described.

The graph for food vacuole formation rises by successive stages to 23.2 vacuoles per individual and then similarly falls to practically the same level (8.8 per individual) as at the beginning of the life-history of this culture. These results find no parallel in the graphs for the contractile vacuoles or the division rate. However, it is significant that minor fluctuations coincide in almost every case with depressions in reproductive activity.

The closely synchronous fluctuations in food-vacuole formation, contractile vacuole rate, and rhythms in the division rate point to some unknown intrinsic factor as holding the key to the problem of rhythms in this species. That temperature is not this crucial factor is apparent by a careful analysis of the temperature curve in figure 7.

Culture II. Culture II of Paramecium calkinsi originated from the same animal which constituted the source of culture I. Observations on this culture were concluded at the one hundredth generation after it had lived synchronously with culture I for eighty days. Figure 8 presents a graphic record of all the phenomena observed in culture II.

These animals were also examined cytologically, but no evidence of endomixis was found nor was there any evident relation between the visibility of the micronuclei and the rhythms in reproductive activity. There is about the same degree of difference in the division rate, as in culture I, between the high and low points: low at 0.4 and high at 2.0.

In this culture, too, the variations in the rates of the vacuoles is more pronounced than in culture I; but even here the greatest difference between the extremes of pulsation rates were never more than 1.3 contractions per minute. The anterior and posterior vacuoles maintained almost an identical contractile rate, as a rule. Slight variations in the rates of the two vacuoles, from day to day, tended to counterbalance one another. This will be discussed later in an account of data from all cultures of this species.

The fluctuations in food vacuole formation are more nearly correlated with fluctuations in contractile vacuole rate and rhythms in division rate here than in culture I. Indeed, when the exigencies incidental to such a long-continued experiment are taken into account, the correlation is really remarkable.

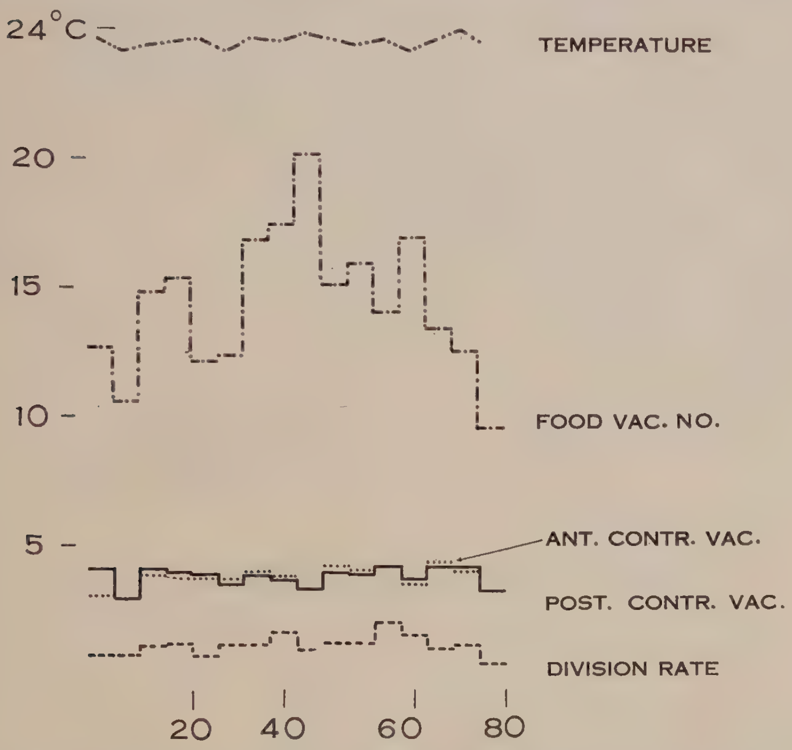

Fig. 8 Record of observations on culture II of Paramecium calkinsi for eighty days plotted at five-day intervals. The ordinates have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure.

The temperature record shows almost no points of similarity with the rhythmical activities of the animals, and, therefore, again obviously, is not a controlling factor in the phenomena under analysis.

Combination of results from cultures I and II. A survey of all data from cultures I and II is afforded by a combination of averages of data presented in figure 9. This treatment of the data is legitimate, since, for all practical purposes, cultures I and II may be regarded as one culture comprising eight lines. It is noticeable that all the curves of vital phenomena show synchronous depressions and elevations, although the fluctuations for food-vacuole formation are more conspicuous than those for contractile vacuole and division rate.

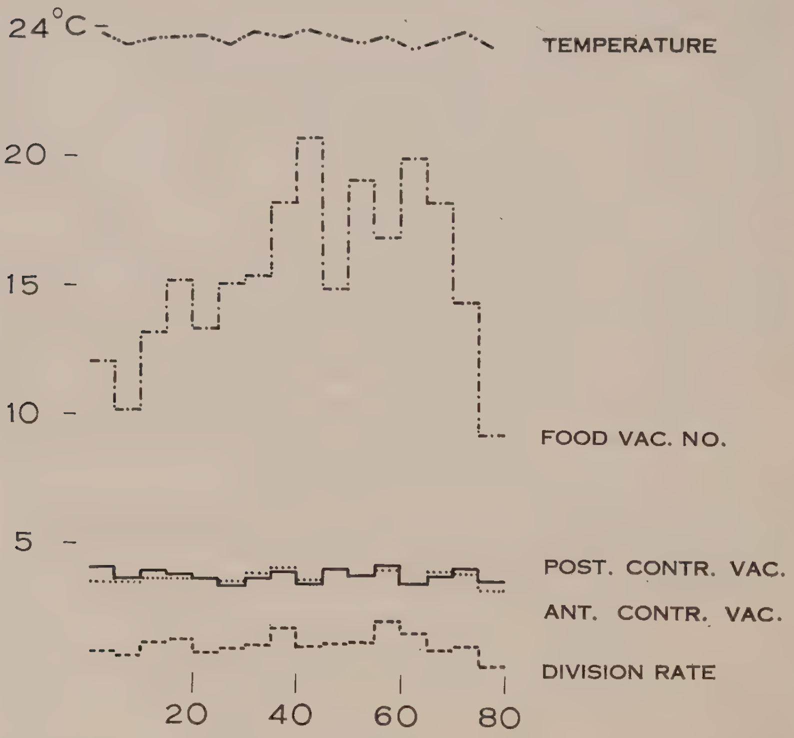

Fig. 9 Combined record of observations on cultures I and II of Paramecium calkinsi for eighty days plotted at five-day intervals. The ordinates have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure.

Culture III. Another culture of Paramecium calkinsi was bred from January 5 to February 13, 1925, and in that time it attained sixty-three generations. The initial animal used to start culture III was taken from a mass culture seeded from an individual at the close of culture II. Therefore, culture III was a continuation of the same race as that used in cultures I and II.

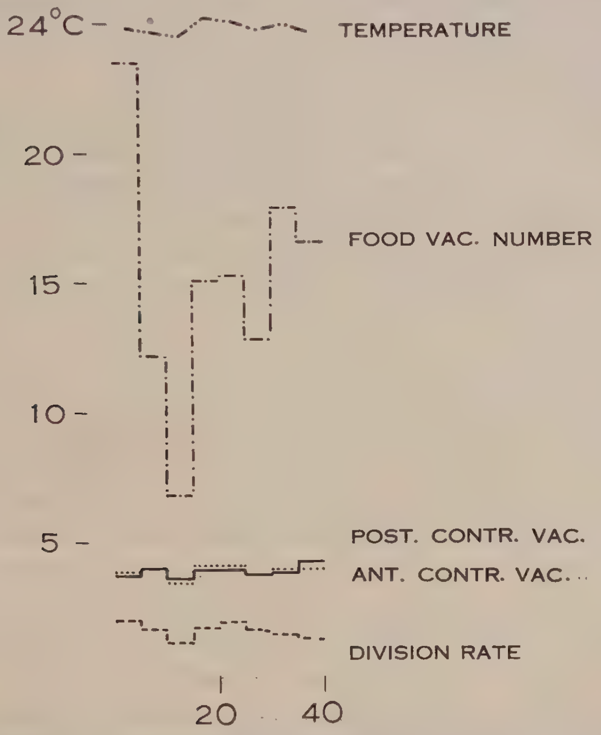

Fig. 10 Record of observations on culture III of Paramecium calkinsi for forty days plotted at five-day intervals. The ordinates have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure.

Figure 10 shows the results of the experiments and observations on this culture. During its forty-day history, two rhythms occurred in the division rate and less marked fluctuations in the excretory activity. In the latter fluctuation, a rise in contractile vacuole rate occurred synchronously with a falling off in the division rate. The food vacuole number at this juncture fell off, then rose, and then again dropped, thus showing no obvious relationship in this instance to the other phenomena of the cell.

At periods fifteen and twenty-five days after the beginning of this culture, the excretory and reproductive activity rose to the same height as existed at the end of the first five-day period, though the food vacuole number did not attain nearly so high a position as it had during the previous high level of the excretory and reproductive fluctuations.

From the study of these three cultures, it is obvious that fluctuations in the division rate, nutritive activity, and excretory activity are present in Paramecium calkinsi when bred under practically constant environmental conditions, although no underlying phenomenon such as endomixis is demonstrable. This seems to be positive evidence that rhythms are not due to endomixis.

The number and size of food vacuoles in Paramecium calkinsi. We may now consider all food-vacuole data collected from all cultures of Paramecium calkinsi during the experiments which extended over 120 days.

Seven hundred and forty-one individuals were examined microscopically and showed 11,506 food vacuoles. Individuals were seen with vacuoles varying in number from one to fifty; there were two of these extremes. A graph of the distribution of the number of food vacuoles per individual is shown in figure 11; the number of vacuoles per individual has been plotted for variations of five vacuoles, because the data at hand were not voluminous enough to iron out irregularities due to minor incidental factors when data was presented for variations of one vacuole per individual. It is seen that there were 185 animals which contained ten to fifteen vacuoles each.

The diameters of the 11,506 gastric vacuoles were measured and the results are presented in figure 12. The smallest seen in Paramecium calkinsi was 2 μ in diameter, and the largest was 20 μ in diameter. There were but five of the former and three of the latter. In all, 6144 vacuoles had a diameter of 4 μ and 3674 had a diameter of 6 μ. Since the majority were 4 or 6 μ in diameter, this may be considered the typical size of vacuoles formed.

Rates of the anterior and posterior contractile vacuoles. Figures 7, 8, 9, and 10 show that there is little difference between the rates of the anterior and posterior contractile vacuoles in this species of Paramecium. Averages from all five-day periods in all three cultures were made, and it was found that the anterior vacuole averaged 3.79 contractions per minute and the posterior averaged 3.81 contractions per minute. These figures (differing by only 0.02) suggested that this slight difference might well be within the limits of probable error of the method.

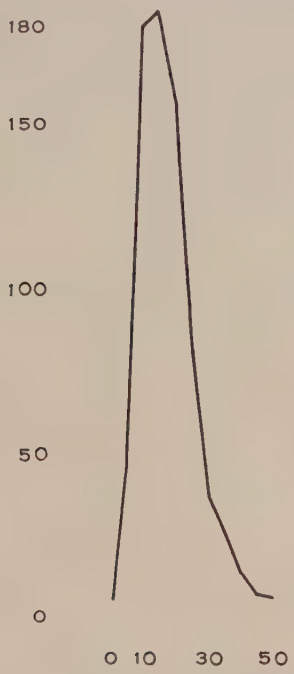

Fig. 11 This graph shows the distribution of the number of food vacuoles per individual in Paramecium calkinsi. The ordinates represent the number of animals and the abscissas represent the number of vacuoles.

Computation of the original observations in seconds showed a mean of 15.65 seconds between contractions of the anterior vacuole and a mean of 15.59 seconds between contractions of the posterior vacuole. Expressed in terms of contractions per minute, these values are equivalent to 3.83 contractions for the anterior and 3.84 contractions for the posterior. The difference in values obtained by the two methods (one on the basis of seconds and the other on the basis of daily averages expressed in contractions per minute and then averaged at five-day intervals) is due to the less exact method used in computing five-day averages whereby decimals beyond tenths were disregarded.

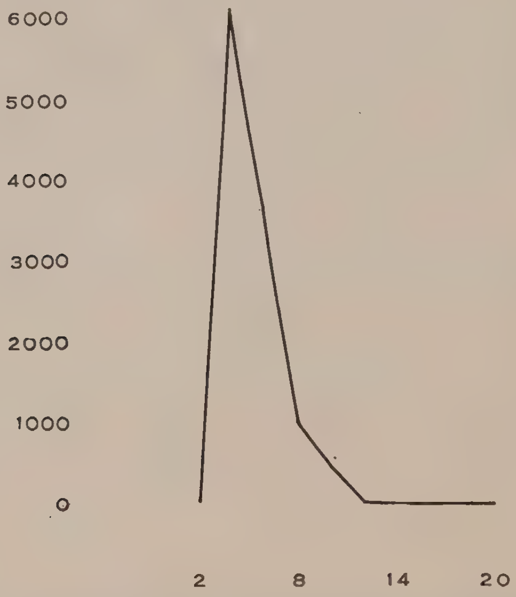

Fig. 12 This graph shows the distribution of 11,506 food vacuoles in Paramecium calkinsi, with reference to their diameters. The ordinates represent the number of vacuoles and the abscissas represent the diameters of the vacuoles in micra.

Data analyzed by the more exact method showed a probable difference of plus 0.042 and an observed difference of minus 0.018 (table 2); which indicates conclusively that there is no significant difference between the rates of the anterior and posterior contractile vacuoles in Paramecium calkinsi. On the assumption that contractile-vacuole rate is an indication of metabolic rate, it must be concluded that the latter does not differ at the anterior and posterior ends of this species.

C. Comparison of Paramecium aurelia and Paramecium calkinsi reared synchronously

The data presented show that a culture of Paramecium aurelia (fig. 4) and one of Paramecium calkinsi (fig. 10) were bred simultaneously for thirty-five days from January 5 to February 8, 1925, under identical conditions except for the slight difference in culture media. In that time, two synchronous occurrences of endomixis and rhythms were demonstrated in Paramecium aurelia and two rhythms in Paramecium calkinsi. Figures 4 and 10 are essentially similar to portions of figures 3 and 9, respectively, which show the history of experiments over eighty days in each species. Figures 4 and 10 show the occurrence of synchronous rhythms; this discounts any possible objection which might be raised because the earlier eighty-day cultures of P. aurelia and P. calkinsi were observed at different times of the year or under slightly different conditions. In other words, these thirty-five day observations compared with the eighty-day observations show that the rhythms of both species exist at all times and afford further evidence that the rhythmical activities are based on intrinsic factors.

TABLE 2

Comparison of contractile vacuole rates in Paramecium calkinsi

	NUMBER OF CONTRACTIONS	RANGE IN SECONDS	MEAN	STANDARD DEVIATION
Anterior vacuole	3397	9 to 35	15.65±.031	2.76±.02
Posterior vacuole	3452	10 to 33	15.59±.029	2.52±.02

Probable difference, +0.042
Observed difference, −0.018

D. Paramecium caudatum

Culture I. The culture of Paramecium caudatum studied was started from one individual isolated on December 20, 1924. From the mass culture started on that day, one animal

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was isolated on January 4, 1925; observations were begun on January 14th and continued fifty-five days until March 11, 1925. During the period of observation, the culture of four lines attained one hundred generations. Figure 13 gives a record of all observations made upon this culture.

The graph for division rate shows two depressions; coincident with the latter, the endomictic process was demonstrated, accompanied by a pronounced depression in all other phenomena observed. Although endomixis occurred only once during the fifty-five-day study, this is not surprising, since Erdmann and Woodruff have shown that endomixis occurs every fifty to sixty days or from eighty to one hundred generations in this species. It was noticed that the appearance of the macronuclear reorganization was coincident with the low point on the curves for contractile vacuole rates. The number of food vacuoles formed also shows the lowest number at this depression point.

The most striking fact demonstrated in this culture is the clear-cut evidence that, on the average, the anterior contractile always pulsated faster than the posterior. There were a few animals observed where the posterior vacuole did pulsate faster than the anterior, but such cases were comparatively rare.

In this species, as in all the others observed, the food-vacuole data show a general similarity to the other curves representing metabolic activity. From the beginning of observations, there was a downward trend to the number of food vacuoles formed, and once this low point was reached there was an increase in the number of food vacuoles formed. The smallest number of food vacuoles per individual at any five-day period was 11.4 and the largest was 21.6.

A study of the graph of such slight temperature fluctuations as occurred shows no definite relationships to the other phenomena under the conditions of the experiment.

The data presented in figure 13 show that fluctuations in the nutritive and excretory activities are present in Paramecium caudatum synchronously with the rhythms in division rate and with the occurrence of endomixis.

The number and size of food vacuoles in Paramecium caudatum. In the history of this culture of four lines of Paramecium caudatum, 218 animals were examined, and they contained 3375 food vacuoles, or an average of 15.46 vacuoles per individual. Two animals had no food vacuoles and two had thirty-three food vacuoles each; these were the extremes in the number of food vacuoles observed in this species. A graph of the distribution of the number of food vacuoles per individual is shown in figure 14; the number of vacuoles have been plotted for variations of five vacuoles per individual. It is seen that there were ninety-seven animals which contained from ten to fifteen vacuoles each.

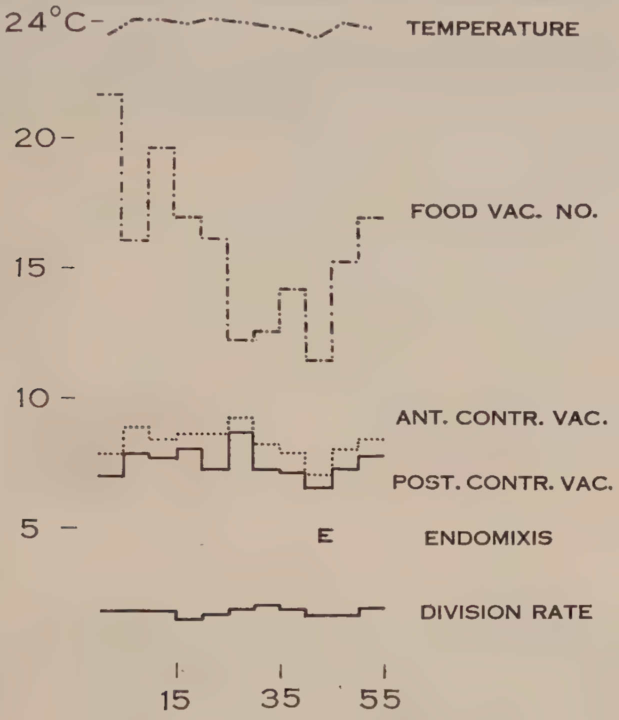

Fig. 13 Record of observations on culture I of Paramecium caudatum for fifty-five days plotted at five-day intervals. The ordinates have the same numerical value for all curves; the abscissas represent five-day periods. The temperature record is shown at the top of the figure. Endomictic periods are designated by the letter *E*.

The size of food vacuoles varies between 4 μ and 24 μ in diameter. There were ten of the former and one of the latter size. Figure 15 clearly shows that the mode is represented by 10 μ, there being 1487 vacuoles with that diameter. The majority (2839) of food vacuoles were 8, 10, and 12 μ in diameter. Although these observations were limited to 218 animals and 3375 vacuoles, the results are derived from a pedigree culture under controlled conditions, and undoubtedly are typical for the race of Paramecium caudatum under observation.

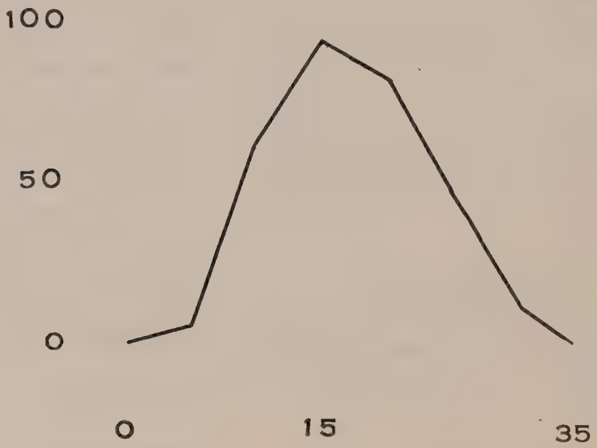

Fig. 14 This graph shows the distribution of the number of food vacuoles per individual in Paramecium caudatum. The ordinates represent the number of animals and the abscissas represent the number of vacuoles.

Rates of the anterior and posterior contractile vacuoles. In figure 13 it was seen that, on the average, the anterior contractile vacuole pulsated more rapidly than the posterior. An average computed from all the data of five-day periods showed that the anterior vacuole contracted 8.27 times per minute and the posterior contracted 7.48 times per minute; or, expressed in terms of difference, the anterior vacuole pulsated 0.79 time more often per minute than the posterior.

There was no doubt that the anterior vacuole contractions were more rapid than the posterior by a sufficient margin to be significant, and an analysis of the data on the basis of the original differences in seconds between beats bears this out. By this calculation, a mean of 7.25 seconds for the anterior and a mean of 8.06 seconds for the posterior was disclosed. When resolved into terms of contractions per minute, this equals 8.27 contractions per minute for the anterior vacuole and 7.44 contractions per minute for the posterior.

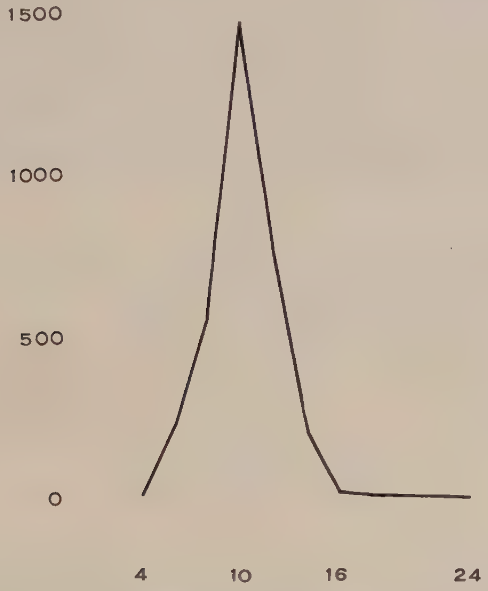

Fig. 15 This graph shows the distribution of 3375 food vacuoles in Paramecium caudatum, with reference to their diameters. The ordinates represent the number of vacuoles and the abscissas represent the diameters of the vacuoles in micra.

It was found (table 3), on the basis of the intervals in seconds between contractions, that the probable difference between the rates of the two vacuoles was 0.012, whereas the observed difference was 0.673 second. Thus the conclusion appears secure that the anterior vacuole pulsates more rapidly, and, if contractile vacuole rate is a fair index of metabolic activity, this may be interpreted as indicative of higher metabolic activity at the anterior than at the posterior end in Paramecium caudatum.

DISCUSSION

From a consideration of the foregoing experiments on Paramecium aurelia, Paramecium calkinsi, and Paramecium caudatum, it is evident that rhythms are not caused by endomixis, since these rhythms are present in P. calkinsi (where endomixis does not occur) as well as in P. aurelia and P. caudatum (where endomixis does occur). Thus, incidentally the work of Woodruff ('21) and of Spencer ('24) is substantiated. Furthermore, all previous work on P. calkinsi has employed the type race at Yale University. In the present study, however, another race, secured at Woods Hole, Massachusetts, was employed, and it is of considerable interest to establish the fact that this race also does not undergo endomixis. Accordingly, it is now safe to assume that the absence of endomixis is not merely a racial, but a specific, characteristic of P. calkinsi.

TABLE 3

Comparison of contractile vacuole rates in Paramecium caudatum

	NUMBER OF CONTRACTIONS	RANGE IN SECONDS	MEAN	STANDARD DEVIATION
Anterior vacuole	1067	5 to 14	7.25±.02	1.12±.01
Posterior vacuole	1058	5 to 15	8.06±.02	1.27±.01

Probable difference, 0.012
Observed difference, 0.673

Of still more interest, however, is the fact that fluctuations in excretory and nutritive activity, synchronous with rhythms of the reproductive rate, are shown to be present (Unger, '25). Apparently these fluctuations in the metabolic activity of Paramecium aurelia are accentuated by the occurrence of endomixis, since the changes in the rate of the contractile vacuoles, in particular, are more abrupt and extend over a greater range than those of Paramecium calkinsi. Since endomixis occurred but once in the culture of Paramecium caudatum while under observation (the intermictic period is longer in P. caudatum than in P. aurelia), no far-reaching conclusions can be drawn for this species, although endomixis is present at the low point on the curves for excretory activity.

A careful study of food-vacuole data reveals the fact that the average number of food vacuoles formed per individual differs in the three species. From observations on the number of food vacuoles in 719 P. aurelia, in 741 P. calkinsi, and in 218 P. caudatum, it was learned that P. aurelia averaged 12.66 food vacuoles per individual, P. calkinsi averaged 15.25 per individual, while P. caudatum averaged 15.46 per individual (table 4).

Reference to figures 6, 12, and 15 and table 4 will show that in P. aurelia the mode of food vacuole size was 10 μ and the mean was 11.36 μ; in P. calkinsi, an examination of 11,506 vacuoles disclosed a mode of 4 μ and a mean of 5.36 μ; in P. caudatum the mode of food vacuole size was 10 μ and the mean was 10.17 μ. Thus the majority of food vacuoles found within the endoplasm of the three species tends to be smaller in P. calkinsi than in P. aurelia and P. caudatum.

TABLE 4

Average number and size of food vacuoles present in three species of Paramecium

	ANIMALS OBSERVED	FOOD VACUOLES OBSERVED	AVERAGE NUMBER FOOD VACUOLES PER ANIMAL	AVERAGE SIZE IN MICRA
P. aurelia......	719	9105	12.66	11.36
P. calkinsi.....	741	11506	15.25	5.36
P. caudatum...	218	3375	15.46	10.17

A critical study of the number of food vacuoles present at the low and high points in rhythms of the different species discloses the interesting fact that all species show an almost identical base level at 11.03 to 11.44 vacuoles per individual (table 5).

However, at the high points in rhythms, coincident with parallel fluctuations in metabolic activity, the number of food vacuoles present shows a great variation; the average number of vacuoles in P. aurelia at such times is 13.72; in P. calkinsi, 18.39; in P. caudatum, 19.60. So it seems that all species are on a par, as it were, at the low points of rhythms and synchronous fluctuations of metabolic activity, and then nutritive activities increase to a varying degree as the respective high levels are reached to such an extent as to establish a difference significant for various species, each of which is represented here by one race.

On the other hand, an analysis of data for vacuole size at the maximum and minimum points of rhythms in each species examined shows that the size of food vacuoles formed in a given species is remarkably constant at all times, at the top or bottom of rhythms or whether few or many vacuoles be formed (table 6).

TABLE 5

Average number of food vacuoles present in three species of Paramecium averaged for the entire period of observation and for high and low points of rhythms

	AVERAGE NUMBER PER INDIVIDUAL FROM ALL DATA	AVERAGE NUMBER AT TOP OF RHYTHMS	AVERAGE NUMBER AT BOTTOM OF RHYTHMS
P. aurelia	12.66	13.72	11.44
P. calkinsi	15.25	18.39	11.03
P. caudatum	15.46	19.60	11.40

For example, in P. aurelia the highest percentage of food vacuoles formed at the high and low points was 10 μ in size, and next highest at 12 and 14 μ. In P. calkinsi most of the vacuoles at both extremes in rhythms were 4 μ in diameter, and vacuoles of larger size occurred on a decreasing scale almost exactly parallel in both extremes.

In P. caudatum there were more vacuoles of 10 μ in diameter formed at both high and low points of rhythms than of any other size; percentages of other vacuole sizes are, on the whole, parallel.

Therefore, it is evident that, although the number of food vacuoles formed varies at the maximum and minimum points on the rhythms of division rate and the fluctuations of nutritive and excretory activity, the size of vacuoles formed at different periods of metabolic activity is remarkably constant. In other words, adjustment to a requirement for more food is not accomplished by the formation of larger vacuoles, but by the formation of more vacuoles of the usual size.

The rates of the pulsating vacuoles differ in the three species studied. Table 7 gives the rates of the anterior and posterior vacuoles in contractions per minute, computed from the original observations of intervals in seconds between contractions of the respective vacuoles.

TABLE 6

Number of food vacuoles present in the three species of Paramecium at high and low points on rhythms of division rate and fluctuations of metabolic activity. Values expressed in percentages

SIZE µ	P. AURELIA		P. CALKINSI		P. CAUDATUM	
	High	Low	High	Low	High	Low
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
4	.09		48.0	54.4		
6	2.8	2.9	37.6	30.8	3.6	6.5
8	10.1	7.7	9.1	9.9	15.9	21.8
10	43.1	35.4	4.1	4.9	48.6	36.6
12	25.3	27.1	.6	.5	21.6	20.2
14	11.5	16.7	.2	.04	8.0	10.3
16	4.7	6.4	.02	.04	.9	3.8
18	1.0	1.5			.6	.5
20	.8	1.3	.05		.2	
22	.1	.1				
24	.04	.1				
26						
28		.09				
30	.05	.09				

The vacuoles of Paramecium aurelia and Paramecium caudatum pulsate more rapidly than do those of Paramecium calkinsi. Possibly this difference is correlated with the difference in size of the individuals of the three species; the races of P. aurelia and P. caudatum are the larger of the three.

These data can be analyzed in another way. If the vacuoles are conceded to be spherical, then the volume of a typical food vacuole can be computed from the mean vacuole diameter in each species. This volume multiplied by the average number of vacuoles present in an individual animal of each species will give some idea of the amount—i.e., volume—of food present in each species under the conditions of the experiment. Accordingly, it was found that the average amount of food material in the vacuoles of a Paramecium aurelia was 0.000,009,730 cu.mm.; in Paramecium caudatum, 0.000,008,490 cu.mm., and in Paramecium calkinsi, 0.000,001,229 cu.mm. There is actually much more food present in Paramecium aurelia and Paramecium caudatum than in Paramecium calkinsi, and probably this may be related to a higher metabolic rate. But, of course, the whole problem is complicated not only by difference in size of the three species, but also by minor size variations which occur from day to day. It is possible that such daily size variations may be correlated with vacuole number, but the amount of work involved to determine the extent of such a correlation would be all out of proportion to the significance of the results obtained and merely incidental to the larger problems under consideration.

TABLE 7

Contractile vacuole rates

	CONTRACTIONS PER MINUTE	
	Anterior	Posterior
P. aurelia	7.69	8.26
P. calkinsi	3.83	3.84
P. caudatum	8.27	7.44

The rates of the two contractile vacuoles in Paramecium aurelia are not equal. From an average of more than 3300 contractions of each vacuole, it is patent that, in the majority of cases, the posterior vacuole contracts more rapidly than the anterior. On the other hand, a study of the contractile vacuole rates in Paramecium calkinsi shows that, on the average, the anterior and posterior vacuoles pulsate at the same rate. Similar calculations on the contractile vacuoles of Paramecium caudatum, based on the observations of individuals from a pedigree culture for fifty-five days' duration, show that the anterior vacuole pulsates more rapidly than the posterior in the majority of cases.

If the data for the contractile vacuole activity are considered from the angle of the actual number of animals in which the anterior or posterior vacuole pulsated more rapidly, it is evident, as shown in table 8, that in 81 per cent of Paramecium caudatum examined the anterior vacuole beat more rapidly; in Paramecium aurelia 60.2 per cent of the animals exhibited greater vacuole activity at the posterior end, while the percentages of animals in Paramecium calkinsi, showing a more active rate for the anterior or for the posterior contractile vacuole, were almost equal.

TABLE 8

The number of individuals of three species of Paramecium with varying rates of the contractile vacuoles. Values expressed in percentages

	P. CAUDATUM	P. AURELIA	P. CALKINSI
	Per cent	*Per cent*	*Per cent*
Anterior vacuole more rapid	81.0	22.2	29.8
Posterior vacuole more rapid	11.0	60.2	31.0
No difference in rates of the two vacuoles	8.0	17.6	39.2

Child ('14) determined the contractile vacuole rates for Paramecium and found the anterior to be more rapid than the posterior; he also made experiments on the susceptibility of Paramecium to KCN and found that the anterior end was more susceptible than the posterior. Since this reaction with KCN is said to be indicative of the rate of metabolic activity, the anterior region was regarded to be more highly differentiated. Presumably Child worked with Paramecium caudatum, although the species is not stated. Furthermore, Bills ('24) demonstrated the existence of 'susceptibility gradients' in Paramecium by blister formation after subjection to alcohol. These blisters were formed first at the anterior end. He states that he used P. aurelia and P. caudatum.

However, on the basis of the present study of the rate of contraction of the anterior and posterior vacuoles of these species of Paramecium, it is clear that each species studied gives a different result. Accordingly, if we may assume that contractile vacuole rate is a reliable index of metabolic rate and, therefore, of physiological 'polarity,' each species differs radically in this regard.

It is important to emphasize that this entire study may be regarded as an intensive analysis of the physiological characters of three species of the genus Paramecium, while admitting the possibility that future work may indicate racial differences within the various species in regard to vacuole rate. Needless to say, in the final analysis morphology and physiology are but reciprocal aspects of the living organism, but morphology for practical reasons is the one which is emphasized as a taxonomic criterion. However, it is equally important that specific criteria should also be based, whenever possible, on physiological characters. And the present study involves an attempt to do this for the species studied.

By this investigation, fluctuations in nutrition and excretion, as indicated by food-vacuole formation and contractile-vacuole pulsation, are shown to be coincident with, and probably related to, the well-established rhythms in the reproductive activity of Paramecium. In other words, the data amassed show conclusively that rhythms in the division rate and fluctuations in the metabolic activity of Paramecium are not intrinsically the result of nuclear reorganization processes, temperature variations, or differences of the food material, but to some unknown, intrinsic, physiological factor in the protoplasm of the cell.

SUMMARY

The following points may be emphasized:

1. Fluctuations in nutrition and excretion as indicated by food-vacuole formation and contractile-vacuole pulsation are coincident with the well-established rhythms in the reproductive activity of Paramecium.

2. Rhythms in reproductive activity are present in Paramecium calkinsi (which does not undergo endomixis) as well as in Paramecium aurelia and Paramecium caudatus (which do undergo endomixis).

3. Under similar conditions, the contractile vacuoles of Paramecium aurelia and Paramecium caudatum pulsate more rapidly than those of Paramecium calkinsi.

4. In the majority of cases in Paramecium aurelia the posterior contractile vacuole beats more rapidly than the anterior. The anterior and posterior contractile vacuoles of Paramecium calkinsi pulsate at the same rate. The anterior contractile vacuole of Paramecium caudatum pulsates more rapidly than the posterior in the majority of cases.

5. The differences of the relative rates of the anterior and posterior contractile vacuoles in these three species may be interpreted as indicating that each species differs from the other two in regard to physiological polarity.

6. On the average, under the conditions of culture, Paramecium aurelia forms 12.66 vacuoles per individual; Paramecium calkinsi, 15.25 gastric vacuoles; Paramecium caudatum, 15.46 food vacuoles per individual.

7. In all three species, more food vacuoles are formed at the top of rhythms than at the bottom.

8. The average size of food vacuoles present in Paramecium aurelia is 11.36 μ in diameter; smallest, 4 μ; largest, 30 μ. Food vacuoles of Paramecium calkinsi are, on the average, 5.36 μ in diameter; the extremes of vacuoles seen were 2 μ and 20 μ. The average size of food vacuoles present in Paramecium caudatum is 10.17 μ in diameter; the smallest vacuoles seen were 4 μ in diameter and the largest were 26 μ.

9. The proportionate number of food vacuoles of a given size formed in a given species is almost identical at the high and low points of curves for nutritive activity.

10. Data are contributed toward a differentiation of three species of the genus Paramecium on the basis of physiological characters, though, of course, it is possible that future work may prove that some of the characters do not transcend racial variation within the species.

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Spiders from the Virgin Islands

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SPIDERS FROM THE VIRGIN ISLANDS

INTRODUCTION

The spider fauna of the Virgin Islands has never been made a subject of thorough study. A single species has been recorded from the British Virgin Islands, and only twelve species, distributed over eight families, have previously been recorded from the American Islands. One of these spiders, *Diplura macrura* C. Koch, belonging to the Family Dipluridæ, has been taken in St. John and later found to occur also in Cuba. Another, *Trochanteria ranuncula* Karsch, belonging to the Family Drassidæ, has been described from St. Croix and never captured again. The remaining ten species have been collected in St. Thomas. Of these *Mygale drassiformis* C. Koch of the Sub-Order Mygalomorphæ cannot be identified, but is probably a *Diplura,* according to Ausserer; *Evophrys venusta* and *Marpissa incerta* of the Family Attidæ have both been insufficiently described by C. Koch, and like the preceding species cannot be identified at present. *Gasteracantha tetracantha* L. of the Family Argiopidæ is widely distributed throughout the West Indies. *Modisimus glaucus* Simon of the Family Pholcidæ has been collected also in Cuba, Hayti, Jamaica and St. Vincent. *Cyrtopholis bartholomei* (Latreille), originally described from St. Bartholomew, has been found in St. Thomas and also in Antigua, if the identification of material from the last-named island is correct. *Cyrtopholis acutispina* Strand and *C. pelus* Chamberlin of the Family Theraphosidæ, and *Odo agilis* Simon of the Family Ctenidæ have been described only from St. Thomas. *Clubiona maritima* L. Koch of the Family Clubionidæ has been recorded since from Cuba. Cosmotropical and neotropical species may have been collected in the Virgin Islands, but have not been recorded.

To interpret these meagre records as indicating any paucity of spiders in the fauna of the Virgin Islands would be a gross error. A little collection of spiders, which Dr. C. R. Shoemaker of the U. S. National Museum made in St. Thomas in 1915 and placed at my disposal, contained no less than 25 species, not counting five species of immature spiders which could not be identified beyond the genus. Eight species proved to be new, three of them representing new genera. My own collections, made during the summer and autumn of 1925, when an opportunity was afforded to visit the principal islands of the group now belonging to the United States, have yielded noteworthy results. These are set forth in the following pages and constitute the second part of my studies of the spider fauna of Central America and the West Indies, commenced in these Transactions, July 1925. As the present article has been largely prepared during my year of service as Visiting Professor of Zoology at the University of Porto Rico, it may properly be regarded as a contribution to science from that institution.

During five days in St. Thomas I collected fifteen species, three of which are new, while seven others are not represented in the collection of Dr. Shoemaker. During two days, spent in St. Croix, I observed and recorded seven cosmotropical or neotropical species, all exceedingly common. *Artema atlanta* Walck. and *Uloborus geniculatus* Oliv. were the common spiders inside buildings, while *Menemerus bivittatus* Duf. and *Plexippus paykulli* (Aud.) could be seen on the outside of the walls in the sunshine. *Filistata hibernalis* Hentz made numerous webs on walls, and at the time of my visit in the first week of September males were rare. *Araneus* (*Metepeira*) *labyrintheus* (Hentz) built innumerable webs between the telephone wires on the streets.

The ecological conditions in the Virgin Islands are in many ways peculiar. The islands are hilly, the hills rising more or less abruptly from the sea, but none are of sufficient height to influence the vertical distribution of the spider fauna, the highest hill in St. Thomas being 1549 feet. St. Thomas is 13 miles long and 4 miles wide. There is no definite information as to whether St. Thomas was ever covered with a forest. At the present time most of the land is hilly pasture with small isolated thickets of second growth and a few larger trees. There is but a single town in St. Thomas, and only small, scattered homes are to be seen elsewhere on the island. The water supply is not abundant, prolonged droughts being common, and rainwater only being available. There is plenty of vegetation for spiders which need no forest. In the town itself, wherever the streets are not paved, and especially on the small lawns of the public square near the harbor, one finds numerous holes in the ground, inhabited by

Cyrtopholis bartholomei, and in some places separated from one another by not more than two feet. Similar holes are found all the way up to the top of the hill, sometimes in very hard soil in the road itself or in the banks on either side of it. On the bushes along the road are numerous webs of *Argiope argentata, Cyclosa walckenaeri* and *Araneus* (*Metepeira*) *labyrintheus,* occasionally also *Gasteracantha tetracantha. Argyrodes nephilæ* is quite common in the nets of *Argiope. Leucauge argyra* is very common, but at the time of my visit (last week of July) most of the specimens were immature. I have not found any specimens of *Latrodectus mactans,* and inquiry among natives showed that they were not acquainted with it. It is therefore very probable that *Latrodectus* does not occur on the island.

The largest of the islands is St. Croix, which is about 21 miles long and 6 miles wide. Lying to the southward of the rest of the group, it is separated from St. Thomas by a deep channel 40 miles wide. St. Croix must have been well forested at one time, but in the year 1650 the forests were burned. Knox[1] writes: "In order to arrest the mortality which was so rapidly thinning their numbers—a mortality which arose from the dense and aged forests that covered the island, scarcely affording an opportunity for the winds to carry off the poisonous vapors with which its morasses clogged the atmosphere—the colonists who remained, set fire to the woods, and going on board their ships, became spectators of the conflagration. They returned on shore after the flames were extinguished." Notwithstanding this and the severe drought of 1661, the island must have again become densely forested, for when the Danes who purchased it from the French arrived in St. Croix in 1735 "they found the shore covered with such a dense growth of bushes, vines and trees, that the party had to remain three days on board their vessel, whilst the slaves cleared sufficient space for them to land and remain with any comfort."[2] At present St. Croix is almost completely deforested. Small patches of trees with second growth thickets may be seen in some of the valleys higher up on the hills and here and there lower down. Most of the low land is under sugar cane cultivation, while the slopes of the hills are covered with pastures. Prolonged droughts are common, as in St. Thomas, and rainwater only is available.

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[1] Knox, John P., A Historical Account of St. Thomas, W. I., 1852, p. 32.

[2] Op. cit., p. 78.

St. Croix is a much larger island than St. Thomas and, although heavily deforested, undoubtedly must possess a spider fauna equal to that of the latter island. I have no doubt that excursions to the remnants of forest and to the small thickets in the hills, places which lack of time prevented me from visiting, would add many species to the list, including possibly some new to science.

Although apparently uninhabited at the time of their discovery by Columbus, the Virgin Islands must have been frequently visited by Caribs. In the seventeenth century St. Thomas became the visiting place of many vessels. "Thus an extensive entrepôt of the productions of almost every country was established, and the island no doubt soon became the resort of trading vessels from all points of the West Indies and South America. . ."[3] Toward the end of the eighteenth century St. Thomas "became the only market in the West Indies for the products of all the colonies, and the only channel through which they could be conveyed to the countries in the North of Europe."[4] Until the Great War the Virgin Islands, and especially St. Thomas, continued to be frequently visited by vessels. This may possibly account for the presence of cosmotropical and neotropical species in these islands.

Apparently nothing except bay rum and rum was formerly produced for exportation by the Virgin Islands. After the enactment of prohibition sugar began to be exported from St. Croix, and almost at the same time some charcoal, made in St. John, began to be imported into Porto Rico. It is difficult to conceive of either of these articles as responsible for the importation of any but cosmopolitan species found in warehouses and around wharfs. There is nothing extraordinary then in the comparatively large proportion of species peculiar to St. Thomas. Other species found on the American Continents or on the Greater Antilles, and now for the first time recorded from St. Thomas, may be provisionally regarded as late importations, in which case there would be nothing extraordinary in their absence from the other Virgin Islands. It is very likely, however, that eventually they will be recorded as occurring there also.

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[3] Op. cit., p. 96.

[4] Idem, p. 100.

LIST OF SPIDERS OF THE VIRGIN ISLANDS

TORTOLA

SUB-ORDER ARACHNOMORPHÆ

Family Thomisidæ

Stephanopis stelloides (Walckenaer).

ST. JOHN

SUB-ORDER MYGALOMORPHÆ

Family Dipluridæ

Diplura macrura C. Koch, has been also reported from Cuba.

ST. CROIX

SUB-ORDER ARACHNOMORPHÆ

Family Uloboridæ

Uloborus geniculatus Olivier, common in buildings.

Family Filistatidæ

Filistata hibernalis Hentz, common on walls of buildings.

Family Drassidæ

Trochanteria ranuncula Karsch, described from St. Croix and never again found there or elsewhere.

Family Pholcidæ

Artema atlanta Walckenaer, common in buildings.

Family Argiopidæ

Araneus (*Metepeira*) *labyrintheus* (Hentz), common on telephone wires.

Araneus (*Neoscona*) *nauticus* L. Koch, common on buildings.

Family Attidæ

Menemerus bivittatus (Dufour), common on walls.

Plexippus paykulli (Audouin), common on walls.

ST. THOMAS

SUB-ORDER MYGALOMORPHÆ

FAMILY THERAPHOSIDÆ

Cyrtopholis bartholomei (Latreille), first described from the island of St. Bartholomew, later from Antigua; is quite common. Female lives in holes in the ground about one to one and one-half feet deep.

Cyrtopholis acutispina Strand.

Cyrtopholis pelus Chamberlin.

Ischnocolus shoemakeri sp. nov., several females and young.

Mygale drassiformis C. Koch, an inadequately described species, possibly a *Diplura* according to Ausserer.

FAMILY BARYCHELIDÆ

Obaerarius insulanus, gen. et sp. nov., a single female.

FAMILY CTENIZIDÆ

Phaeoclita fauna Simon, 2 females. Originally described from Venezuela.

SUB-ORDER ARACHNOMORPHÆ

FAMILY ULOBORIDÆ

Miagrammopes ciliatus sp. nov., a single female.

Uloborus geniculatus Olivier, several females from Blue Beard's Castle. Cosmopolitan species.

FAMILY FILISTATIDÆ

Filistata hibernalis Hentz, several females. Pan-American species.

FAMILY OECOBIIDÆ

Oecobius parietalis Hentz, several females from Sta. Maria Bay, found under little webs on tree trunks and rocks. Occurs in the Southern United States of America, Baja California and in Mona, W. I. I have numerous specimens from Guadeloupe and Porto Rico.

FAMILY DYSDERIDÆ

Ariadna arthuri sp. nov., a single female from Sta. Maria Bay.

FAMILY SICARIIDÆ

Loxosceles rufipes (Lucas), a single female. Neotropical and subtropical species.

Scytodes fusca Walckenaer, 2 females in Dr. Shoemaker's collection and 2 females from Sta. Maria Bay, found under bark of tree trunk lying on the ground. Neotropical species. Reported from the following islands in the Antilles: Cuba, Hayti, Jamaica, Porto Rico, Mona, Dominica, St. Vincent. Occurs also in Bermuda.

FAMILY PHOLCIDÆ

Artema atlanta Walckenaer, several males and females. Neotropical species. Reported from Porto Rico and St. Vincent. I have specimens from St. Croix and Guadeloupe.

Modisimus glaucus Simon, a West Indian species reported from Cuba, Hayti, Jamaica and St. Vincent.

FAMILY THERIDIIDÆ

Argyrodes nephilæ Taczanowski, numerous females and males from webs constructed on webs of *Argiope argentata* F. Neotropical and subtropical species reported from Hayti, Mona and Desecheo in the Antilles.

Spintharus minutus sp. nov., a single female in Dr. Shoemaker's collection.

Theridion tepidariorum C. Koch, a cosmopolitan species.

FAMILY ARGIOPIDÆ

Araneus (Metepeira) labyrintheus Hentz, numerous males and females. A pan-American species.

Araneus (Neoscona) nauticus L. Koch, a cosmotropical species.

Argiope argentata Fabricius, neotropical and subtropical species.

Cyclosa walckenaeri (Cambridge), numerous females and 2 immature males. A neotropical species occurring in Brazil, Colombia, Costa Rica, Guatemala, Mexico, Cuba and Hayti.

Gasteracantha tetracantha L., common. I have in my collection one male and female captured in the act of mating. An Antillean species recorded also from California.

Leucauge argyra (Walckenaer), common; a neotropical and subtropical species.

Leucauge mandibulata F. Cambridge, several males and females in Dr. Shoemaker's collection. Described from Tobasco, Mexico.

Leucauge regnyi Simon, several females; first described from St. Vincent. In my collection I have specimens from Jamaica, Porto Rico and Guadeloupe.

Micrathena militaris F., 2 females in Dr. Shoemaker's collection. First described from Surinam and Cayenne.

Micrathena sagittata Walckenaer, 2 males and an immature specimen in Dr. Shoemaker's collection. Occurs in U. S. A., Mexico, Central America and Ecuador.

Nephila clavipes L., several males and females. A neotropical and subtropical species.

FAMILY SELENOPIDÆ

Selenops lindborgi sp. nov., 1 female and one immature specimen from Sta. Maria Bay, found under bark.

FAMILY SPARASSIDÆ

Heteropoda venatoria L., a cosmotropical and subtropical species.

Olios fasciculatus Simon, one female in Dr. Shoemaker's collection. Occurs in southern United States, Utah, Mexico.

FAMILY CLUBIONIDÆ

Clubiona maritima L. Koch, reported also from Cuba.

Corinna humilis (Keyserling), 4 females in Dr. Shoemaker's collection. An Antillean species described from Cuba, Hayti, Porto Rico, St. Kitts, Dominica and Grand Cayman.

Corinna cleonei sp. nov., 1 female from Sta. Maria Bay, found under bark.

Eutychuroides fuscus gen. et sp. nov., 2 females and an immature specimen in Dr. Shoemaker's collection.

Gayenna sp.?, 1 immature specimen in Dr. Shoemaker's collection.

Stasina sp.?, 2 immature specimens in Dr. Shoemaker's collection.

Syrisca hirsuta Petrunkevitch, 1 male and 2 immature specimens. Originally described from Panama.

FAMILY CTENIDÆ

Odo agilis Simon.

FAMILY PISAURIDÆ

Thaumasia sp.?, 2 immature specimens in Dr. Shoemaker's collection.

FAMILY LYCOSIDÆ

Lycosa sancti thomasi sp. nov., 1 female and 1 immature specimen in Dr. Shoemaker's collection.

FAMILY ATTIDÆ

Corythalia iridescens sp. nov., numerous females and males in Dr. Shoemaker's collection.

Corythalia sp.?, several immature specimens in Dr. Shoemaker's collection.

Evophrys venusta C. Koch, an inadequately described species.

Marpissa incerta C. Koch, an inadequately described species.

Menemerus bivittatus (Dufour), a cosmotropical species.

Plexippus paykulli (Audouin), a cosmotropical species.

Stridulattus stridulans gen. et sp. nov., a single male in Dr. Shoemaker's collection.

Considering the small size of the island of St. Thomas and the uniformity of conditions, as well as the fact that collecting was done in various parts of the island, the above list may be regarded as fairly representative of the spider fauna of St. Thomas. In examining this list and omitting poorly described species and records of immature specimens, we find that of the six species of Mygalomorph spiders four are indigenous, one occurs in Venezuela and one in the Leeward Islands. Of spiders belonging to the Sub-Order Arachnomorphæ six species are cosmopolitan or cosmotropical, nine species are neotropical, neotropical and subtropical or Pan-American, four species occur in Central America and North America as far as the southern states, five species are Antillean and nine are indigenous to St. Thomas. Only one species, *Micrathena militaris*, has been originally described from South America and never reported from any other countries than Surinam and Cayenne. This species, mentioned in Reimoser's Monograph of the genus Micrathena, has been therefore rediscovered after a lapse of almost a century. Its careful description is given below. As a record for comparison it has no value, especially since the habitat of the original specimens is given only by Olivier and errors at that time were quite frequent.

An analysis of the spider fauna of St. Thomas will be given later. There is close relationship between this fauna and that of Porto Rico, but of course, the latter is much richer, as might be expected. One fact stands out clearly, that *the spider fauna of St. Thomas shows distinct relationship to the tropical and sub-*

tropical fauna of North America and Central America, and very little to that of South America.

It gives me pleasure to express here my thanks to Dr. C. R. Shoemaker for the collection which he placed at my disposal, and to Mr. Arthur E. Lindborg, Director of Education in the Virgin Islands, U. S. A., for his help in arranging excursions to different parts of the island of St. Thomas and in actual collecting of specimens.

DESCRIPTION OF SPECIES

SUB-ORDER MYGALOMORPHÆ

FAMILY THERAPHOSIDÆ

Cyrtopholis bartholomei (Latreille) (Figure 1). No detailed description of this species so common in St. Thomas has ever been given and the male is still unknown. I have seen a specimen in Arthur Lindborg's collection, but unfortunately it was preserved dry and was in such poor condition that further study would have been useless.

The females vary rather considerably in size, but much less in their color, which in life is dark greyish-brown with light stripes on legs. The mouth of the burrow in which this species lives is perfectly round, from one to one and a half inches in diameter, and is not protected by any web. The following description refers to a female of an average size.

Female. Total length with cheliceræ 35.0 mm. Carapace 13.5 mm. long, 11.2 mm. wide, flat, truncated anteriorly, distinctly emarginate behind. Thoracic groove transverse, deep, procurved, situated less than one-third from posterior margin. Sides of head distinctly limited by the cephalothoracic sulci. Posterior limit of head not so clear, but evidently narrower than thoracic groove and somewhat in advance of it. Eyetubercle high, almost circular, its transverse axis a little longer than its longitudinal axis in ratio 50:48. The tubercle is almost marginal in position. Eyegroup transversely rectangular in ratio 50:29. First row of eyes procurved, second recurved (Figure 1). Ratio of eyes AME : ALE : PME : PLE = 13 : 13 : 6.5 : 11. Pupil of AME has a diameter 9/13 that of the latter. PME are irregular in outline, angular, white iridescent. All other eyes are transparent. Quadrangle much wider behind than in front, much wider than long in ratio 34:20. Laterals separated by 8/13 of the diameter of ALE. A group of bristles on eyetubercle in front of AME, between them and behind them. Clypeus and margins of carapace fringed with long white hair forming a well-defined border all around the carapace. Four brown bristles on edge of clypeus in front of the eyegroup. A few dark bristles in a middorsal row extending from eyegroup almost to thoracic groove. Surface of carapace clothed with recumbent hair of two kinds, short dark and long white. *Chelicerœ* powerful, without rastellum, clothed with white hair and bristles on dorsal surface except near base where the chitin is completely bare. Promargin with thin scopula followed by a row of 12 teeth, retromargin without teeth, but with heavy fiery red scopula. Stridulating organs wanting. *Lip* wider than long in ratio 70:50, convex, with straight, obliquely converging sides, procurved base and emarginate end. The cuspules occupy almost entire width of lip at end, where they form from three to five transverse rows. *Maxillæ* with parallel sides and rather sharply produced inner distal angle. The heavy, fiery red scopula occupies the entire proedge. Numerous cuspules extend from proximal inner angle to half the width of the segment and about as far distally, forming a more or less triangular field. Trochanter inserted at end. Maxillæ clothed with long, curved, erect brown bristles and with shorter hair of the same type. *Sternum* almost as wide as long, clothed with short grey and long brown, erect hair. The short hair is so numerous that it hides effectually the sigilla. Anteriorly the sternum is narrowed to width of lip, posteriorly pointed in front of fourth coxæ which are contiguous. First coxæ as far apart as third coxæ, but slightly less than second coxæ. *Legs* 4123.

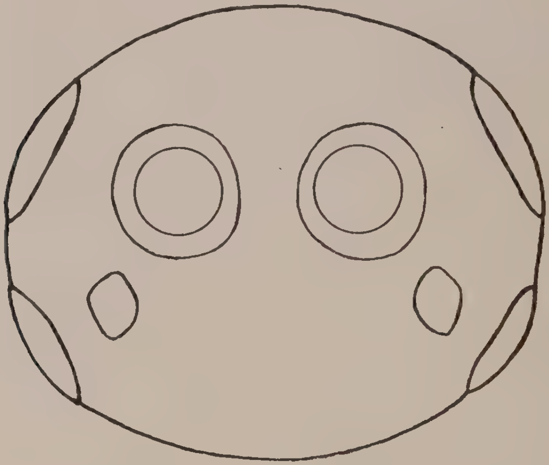

FIGURE 1, *Cyrtopholis bartholomei* (Latreille). Eyegroup of female.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	10.560	6.144	8.560	5.568	4.800	35.632
II	9.408	5.280	6.720	6.240	4.800	32.448
III	8.448	4.800	5.280	6.720	5.280	30.528
IV	10.560	5.568	8.160	9.792	5.280	39.420
Palp	8.256	4.224	5.760	6.240		24.480

No pad or brush on any femora, but the prolateral surface of the first and second femora and the retrolateral surface of the third and especially of the fourth femora flattened and clothed with very short hair. All tarsi scopulate to base, scopulæ very thick, those of fourth pair longitudinally bisected by a narrow band of setæ. First and second metatarsi scopulate to base, third metatarsi scopulate in distal half. Fourth metatarsi with a retro-scopula only, at end of segment. Fourth femora slightly curved laterally, so that the retrolateral flat surface is somewhat concave longitudinally. Terminal joint of palp scopulate to base, its scopula entire. Spines on legs numerous, rather dark. Hair on legs of three kinds, with intermediate types: a) recumbent, short, fine grey-brown hair covering all segments in a more or less even manner; b) recumbent white hair, somewhat longer than the preceding and limited in distribution to the two longitudinal dorsal lines on femora, patellæ and tibiæ of all four pairs of legs; the same type of hair is also present on both lateral surfaces of all femora, patellæ and tibiæ, at the end of tibiæ where it forms a fringe, and in the short middorsal line at base of all metatarsi; c) brown, bristle-like hair of varying length. A few club-shaped hairs on the dorsal surface of all tarsi. Same type of hair on palpi which also have two white dorsal lines on femur, patella and tibia. Ungual tufts thick on all tarsi. Claws two, slightly dissimilar. Those of the first and second tarsi strongly curved, with three teeth which are sharp, well-developed and almost contiguous at base. Those of the third and fourth tarsi much less curved, possessing but two teeth which are separated from each other by a considerable space. Palpal claw strongly curved, with three sharp teeth of the same type as in the claws of the first and second pair of legs. Trichobothria in two rows on all tibiæ, in two irregular rows on all metatarsi and in several rows on all tarsi.

Spines. First leg. Femur—prolateral 1 at end, elsewhere 0. Patella 0. Tibia—dorsal 0, prolateral 1 in middle, retrolateral 0, ventral 1-3, the first in middle, the three apical. Metatarsus—ventral 1 apical, elsewhere 0. *Second leg* same as first, except: Tibia—prolateral 1-1, the second spine near end. Metatarsus—prolateral 1-1, first spine at base, second in middle; ventral 1-3, first spine at base, the other three spines apical. *Third leg.* Femur—retrolateral 1 at end, elsewhere 0. Patella—prolateral 1, elsewhere 0. Tibia—dorsal 0, prolateral 1-1-1, retrolateral 0-1-1, first spine in middle, ventral 2-1p-3. Metatarsus—dorsal 0, prolateral 1-1-1, retrolateral 0-1-1, first spine in middle, ventral 1r-1p-3. *Fourth leg* same as third, except: Patella—0. Tibia—prolateral 0-1-0, retrolateral 1-1-1. Metatarsus—retrolateral 1-1-1, ventral 1r-2-1r-2-1r-2-4. *Palp.* Femur—0. Patella—0. Tibia—dorsal 0, prolateral 1 in middle, retrolateral 1 at end, ventral 1p-1p-4. Terminal point—0.

Abdomen 15.0 mm. long, 10.5 mm. wide, clothed with long hair, short hair and long stiff bristles. First pair of spinnerets very short, only a little longer than the width of the basal joint of the second pair. Second pair of spinnerets, first joint 2.88 mm., second joint 1.92 mm., third joint 1.92 mm. Back of abdomen with a round black spot occupying entire posterior half. This is the common tarantula of St. Thomas. It has much resemblance to the common Porto Rican species, but the latter is much easier excited and more ready to fight.

Ischnocolus shoemakeri sp. nov. (Figures 2 and 3).

Female. Total length with cheliceræ 27.0 mm. Carapace 10.368 mm. long, 8.064 mm. wide, flat. Thoracic groove transverse, straight, deep, situated 6.528 mm. behind anterior edge, *i. e.* approximately one-third from posterior edge. The latter is strongly emarginate in middle, but this notch has not been taken into account in the measurement of the length of the carapace. Anterior edge of carapace straight, 4.32 mm. wide, *i.e.* only about half as wide as carapace in middle. Eyegroup very little elevated, transversely rectangular, with a ratio of length to width 20:44. First row of eyes straight (Figure 2), second row slightly recurved. PME rounded but not circular. They are of the nocturnal type. Ratio of eyes AME : ALE : PME : PLE = 9 : 11 : 8 : 9. AME separated from each other by 6/9 of their diameter, from ALE by 3/9 of their diameter. PME separated from each other by 18/8 of their diameter, from PLE by 1/8 of their diameter, from ALE by 6/8 of their diameter. Laterals separated by 3.5/9 of the diameter of PLE. Quadrangle wider behind than in front in ratio 32:22, wider than long in ratio 32:17. Clypeus equal to 7/9 of the diameter of AME. Width of eyegroup 1.628 mm. Clypeus fringed with brown bristles, sides of carapace with white, woolly hair and white bristles, posterior edge with white hair and brown bristles. A group of bristles on clypeus in front of AME and on the eye-area in the field between the four medians. Carapace clothed with short white recumbent hair and short brown hair. The distribution of the hair is such that there is general appearance of radiation in all directions from the thoracic groove. *Cheliceræ* free from hair at base above. Basal joint 5.28 mm. long, without either rastellum or lyra. Retrolateral surface glabrous, dorsal surface except at base, clothed with white hair and brown bristles. Scopulæ on both margins, light orange in color, the one on retromargin by far the thickest. Promargin with 12 black sharp teeth; retromargin smooth, but a series of 6 minute red-brown teeth or granules between the two margins in a line occupying the space corresponding to the three proximal teeth of the promargin. Fang dark red-brown, rather long and slender. *Lip* wider than long in ratio 44:28, not articulated to maxillæ, but with distinct articulation to sternum on sides where the lip forms a projection and the sternum a socket. Base of lip strongly procurved and surface convex. Sides straight, converging. Anterior edge emarginate, much shorter than base. About eight irregular, transverse rows of cuspules occupy the anterior two-fifths of the lip, leaving free a narrow edge in front and a wider space on each side. Heavy brown bristles in front, on sides and behind the area of cuspules. *Maxillæ* slightly dilated at base, forming

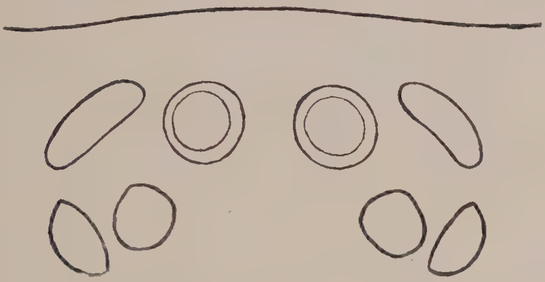

FIGURE 2, **Ischnocolus shoemakeri** sp. nov. Eyegroup of female.

a basal retroprojection or rounded lobe. Scopula of the same color as that on the cheliceræ, occupies entire length of inner edge. Numerous cuspules occupy a field extending at base from scopula to the base of the lobe and almost over half the length of the maxilla, becoming gradually more scattered distally. *Sternum* oval, emarginate anteriorly to accommodate the lip, widest between first and second coxæ, almost but not quite as wide between second and third coxæ, longer than wide in ratio 5/4, clothed with erect, brown bristles which are especially heavy along the margins. Three pairs of submarginal, poorly visible sigilla situated to the outside of the bristles. First coxæ wider apart than third coxæ. Second coxæ widest apart. Fourth coxæ subcontiguous. No peculiar pad or brush of hair on any leg. *Legs* 4123.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	7.680	4.992	5.760	5.184	2.972	26.588
II	6.528	4.224	4.896	4.800	2.972	23.420
III	5.952	3.456	4.416	5.952	2.972	22.748
IV	8.448	4.416	6.912	9.216	4.032	33.024
Palp	5.760	3.360	3.840	3.360		16.320

Legs clothed with recumbent light colored hair and pointed bristle-like, brown hair. Spines numerous, long, dark brown. All tarsi scopulate to base, those of the first pair entire, the others bisected longitudinally by a broad band of setæ. First and second metatarsi scopulate almost to base. Third metatarsi lightly scopulate in distal two-thirds, fourth metatarsi lightly scopulate in distal half. All metatarsal scopulæ, except those of the first pair, longitudinally bisected by setæ. These setæ form a single row on the second metatarsi, two rows on the third, three rows on the fourth. Ungual tufts heavy, completely concealing claws. Claws two, curved, long, with a series of five teeth which are so arranged that they occupy a proventral line on the proclaw (Figure 3) and a retroventral line on the retroclaw. A median basal sclerite present, but third claw wanting. Palpal claw smooth. Terminal joint of palp with entire scopula extending to base. Trichobothria numerous, on tarsi in four rows, on metatarsi somewhat irregular, on tibiæ in two rows. Club-shaped hairs on dorsal surface of tarsi present.

Spines. First leg. Femur—dorsal 0, prolateral 1 toward end, retrolateral 1 toward end, ventral 0. Patella 0. Tibia—prolateral 1-1, ventral 1r-1r-2, elsewhere 0. Metatarsus—ventral 1r-1, elsewhere 0. *Second leg* same as first, except: Femur—dorsal 1-1 near base, prolateral 1-1, first spine one-third from distal end, retrolateral 0. Tibia—ventral 1-1-3. *Third leg.* Femur—dorsal 1-1-2-2-2, prolateral 0, retrolateral 1-1, first spine one-third from distal end, ventral 0. Patella—prolateral 2 in middle, elsewhere 0. Tibia—dorsal 0, prolateral 1d-1v-1d, retrolateral 1d-1v-1d, ventral 1-1-3. Metatarsus—dorsal 0, prolateral 1-1-1, retrolateral 1v-1d-1v-1d, ventral 2-2-3. *Fourth leg* same as third, except: Femur—dorsal 1-1-0-2-2, retrolateral 0, Tibia—prolateral 2-1-2, retrolateral 1-2-1, ventral 2-2-3. Metatarsus—prolateral 2-2-2-2, retrolateral 2-2-2, ventral 1r-1p-1r-1p-3, (metatarsus has altogether 21 spines of which 9 are visible in profile from prolateral side and 11 from retrolateral side). *Palp.* Femur—prolateral 1 toward end, elsewhere 0. Patella 0. Tibia—prolateral 1, ventral 2-2-2, elsewhere 0. Terminal joint 0. *Abdomen* 12.7 mm. long, 6.5 mm. wide, clothed with recumbent short brown hair anl longer bristle-like brown hair. First pair of spinnerets small, slender, cylindrical, composed of a single segment 0.451 mm. long, 0.123 mm. in diameter. Second pair of spinnerets three-jointed when viewed from below, four-jointed when viewed from above. The first joint is 2.540 mm. long, (each component being 1.270 mm. long), the second joint 1.575 mm., the third 1.660, giving the total length for the posterior spinnerets 5.775 mm. *Color in alcohol:* generally rufous, abdomen somewhat greyish, spinnerts yellowish-brown. Field note of Dr. Shoemaker: "found under stones and logs in a ravine. July 1915." Several females and immature specimens from St. Thomas.

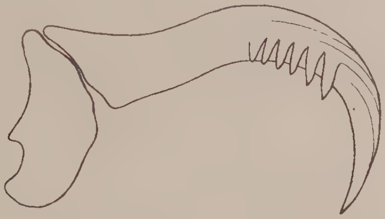

FIGURE 3, **Ischnocolus shoemakeri** sp. nov. Proclaw and basal sclerite of second left leg of female.

FAMILY BARYCHELIDÆ

Obaerarius gen. nov.

Eyegroup on a tubercle, wider than long, transversely rectangular. Eyes of first row equal in size and equidistant, strongly procurved. Second row gently recurved, PME wide apart, but subcontiguous with AME and PLE. Laterals separated by more than their radius. Thoracic groove straight, transverse. Lip wider than long, immobile, spinulose. Cheliceræ, without rastellum, but with numerous spine-like bristles. Maxillæ spinulose. First and second pair of legs almost without spines, third and fourth pair with numerous spines. All tarsi scopulate to base, scopulæ of third and fourth pair broadly bisected by a band of setæ. Spinnerets four, those of the second pair stout, with short terminal joint. Type **O. insulanus.**

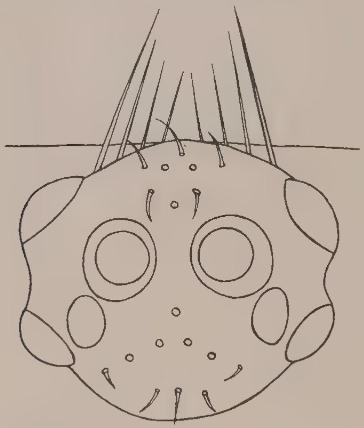

FIGURE 4, **Obaerarius insulanus** gen. et sp. nov. Eyegroup of female.

Obaerarius insulanus sp. nov. (Figure 4).

Female. Total length with cheliceræ 16.0 mm. Carapace 6.912 mm. long, 5.760 mm. wide, flat, widest in middle, truncated in front, slightly emarginate behind. Thoracic groove transverse, straight, deep, situated exactly one-third from posterior margin. Eye-tubercle (Figure 4) steep in front and behind, distinctly angular, marginal in position. Eyegroup transversely rectangular, wider than long in ratio 32: 18. First row of eyes strongly procurved, second recurved. AME subcontiguous with PME, and these with PLE. Ratio of eyes AME : ALE : PME : PLE = 8.5 : 8.5 : 6 : 7.5. AME separated from each other by 3/8.5 of their diameter, from ALE by the same distance. Laterals separated from each other by 6/8.5 of the diameter of ALE. Quadrangle wider behind than in front in ratio 22.5 : 18, wider than long in ratio 22.5 : 12. A transverse row of eight bristles on anterior edge of eyetubercle. Eight bristles in front of AME and ten bristles behind eyegroup on eyetubercle, as shown in Figure 4. A row of bristles in middorsal line between eyetubercle and thoracic groove, terminating in a pair. Five rows of bristles radiating from thoracic groove toward posterior edge of carapace. Anterior edge of carapace devoid of hair. Lateral margins clothed with light colored hair; further back bristles are increasing in number and length. Several bristles opposite third pair of coxæ are especially stout. Surface of carapace clothed with recumbent light colored hair which is sufficiently sparse to leave the color of the chitin clearly visible, dominating the general coloration of the carapace. *Cheliceræ* powerful. Basal joint 3.84 mm. long, clothed with short recumbent white hair and with stiff bristles on dorsal surface. A true rastellum wanting, but the bristles assume the character of very sharp spines toward the end of the basal joint, are here quite numerous and more or less equally spaced. Promargin with 9 or 10 strong black teeth forming a row. Promarginal scopula scanty. Retromargin smooth. Retromarginal scopula well developed, orange. An irregular row of about 8 small teeth between the two margins in the space corresponding to the proximal four teeth of the promargin. Fang long, evenly curved. *Lip* very clearly set off from the sternum, but nevertheless immobile and without intersegmental suture. Its midventral line forms an angle of about 60° with the midventral line of the sternum. Base of lip semicircular, anterior end emarginate. Lip wider than long in ratio 38 : 24. End of lip densely covered with cuspules occupying the field free from bristles. *Maxillæ* less than twice as long as wide, produced at base into a lobe and with a slight lobe at inner distal angle. Scopula well developed, orange. Numerous cuspules at base, leaving free the lobe which is clothed with the same kind of brown bristles as the rest of the maxilla. *Sternum* longer than wide in ratio 9 : 6, almost rectangular in appearance, because the coxæ of the first, second and third pair of legs are about equidistant. Fourth coxæ contiguous, separated only by the short posterior angle of the sternum. Sternum clothed with recumbent short grey hair and stiff erect brown bristles. Sigilla 6. First and second pair of sigilla concealed by the coxæ which hide the edge of the sternum. Third pair round, submarginal, opposite base of third coxæ. All coxæ clothed in the same manner as the sternum. *Legs* clothed similarly, but bristles so predominant that the hair is noticeable only on close examination. Two longitudinal bald stripes on the dorsal surface of all femora, continuing obliquely to end of patella. Tibiæ also with bald stripes, but less noticeable. Trichobothria numerous, on tibiæ, metatarsi and tarsi, but their arrangement is not regular. Club-shaped hairs on dorsal surface of all tarsi. Ungual tufts well developed. All tarsi scopulate to base. Scopula of first tarsus entire, of second tarsus with a few setæ, of third and especially of fourth tarsus longitudinally bisected by a band of setæ. First and second metatarsi scopulate almost to base, with some bristles on second metatarsus. Third metatarsus scopulate in distal half, scopula bisected by setæ. Fourth metatarsus barely with an indication of a scopula at the end, with a band of setæ. Claws 2, of the same type as in *Ischnocolus shoemakeri,* with basal median sclerite, but with only 4 teeth. Femur of pedipalp strongly curved laterally, so that its prolateral surface is longitudinally concave. First and second femora curved in the same sense, but not as much. Fourth femora curved similarly, but so that it is the retrolateral surface which is concave. *Legs* 4123.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	4.320	3.264	2.880	2.400	1.248	14.112
II	4.032	2.880	2.400	2.400	1.248	12.960
III	3.840	2.400	1.920	2.976	1.632	12.768
IV	4.800	2.784	3.072	4.800	2.304	17.760
Palp	3.360	2.112	1.536	2.016		9.024

Spines. First leg. Femur—prolateral 1 at end, elsewhere 0. Patella 0. Tibia—ventral 1-1 bristles, elsewhere 0. Metatarsus—ventral 1-1, elsewhere 0. *Second leg* same as first. *Third leg.* Femur—dorsal 0, prolateral 1 at end, retrolateral 1 at end, ventral 0. Patella—prolateral 2, elsewhere 0. Tibia—dorsal 0, prolateral 2-2-2 rather irregularly arranged, retrolateral 1-1, ventral

2-2-2 not quite regularly arranged and moreover first retrospine and second prospine having more the appearance of bristles. Metatarsus—dorsal 0, prolateral 1-1-1, retrolateral 1d-2-2, ventral 2-2-2. *Fourth leg* same as third except: Femur prolateral 0, Patella—prolateral 1. Tibia—retrolateral 1-1-1. Metatarsus—prolateral 1-1-1-1, retrolateral 2-2-2, ventral 2-2-1p-2. *Palp.* Femur 0, Patella 0, Tibia—prolateral 1-1 first near end, second apical; ventral 1-2, elsewhere 0. Terminal joint 0. Palpal claw with a single basal tubercle, otherwise smooth. Terminal joint of palp scopulate to base, its scopula entire. *Abdomen* clothed with hair and bristles. First pair of spinnerets very slender, second pair stout, three-jointed; their terminal segment a little shorter than the median segment and each of the three segments shorter than their respective diameters. *Color in alcohol:* Carapace, sternum and all appendages rufous. Abdomen greyish-brown with an indistinct yellow middorsal band with two pairs of also indistinct lateral branches. A single female from St. Thomas, collected by Dr. Shoemaker in July 1915.

FAMILY CTENIZIDÆ

Phaeoclita fauna Simon (Figures 5 and 6).

It is possible that the specimen described here is a new species and not *P. fauna* which is twice as long and otherwise structurally slightly different. Nevertheless the difference in size and structure did not seem to me of sufficient importance to necessitate the formation of a new species. Provisionally at any rate, the specimen from St. Thomas may be considered as belonging to the same species with the specimens described by Simon from Venezuela, his description being rather short. The detailed description given here may some day help to differentiate the species of St. Thomas from that of Venezuela.

Female. Total length with cheliceræ 7.296 mm. Carapace 2.952 mm. long, 2.009 mm. wide in middle, 1.640 mm. wide in front, 1.312 mm. wide behind, more or less of the shape of a hexagon. Anterior edge slightly convex, posterior edge slightly concave with median notch. Head elevated and clearly marked off by the cephalothoracic sulci which converge in obliquely straight lines merging with the deep semi-circular, line-like, procurved thoracic groove. The vertex of the groove is exactly one-third from posterior margin. Eyegroup occupies less than one-third of the width of the carapace in that region and is transversely rectangular and but slightly elevated (Figure 5). Both rows of eyes slightly recurved when viewed from above. Viewed from in front first row appears slightly but distinctly curved downward. Ratio of eyes AME : ALE : PME : PLE = 3 : 5 : 3.5 : 3.5. Anterior eyes are about equidistant, separated from each other by 1/3 of the diameter of AME. PME subcontiguous with PLE, but distinctly separated from them by a black line. Similarly the laterals are sub-contiguous, while the ALE are separated from the PME by 1.5/5 of their diameter. Quadrangle wider than long in ratio 11 : 7, wider behind than in front in ratio 11 : 7. Clypeus equal to 2/3 of the diameter of AME. There is no hair on the carapace and only a few bristles. Three bristles in a transverse row on edge of clypeus in front of AME, and three rows of seven bristles each extending from eyegroup to thoracic groove. Of these three rows the median one is composed of much heavier bristles than the others and moreover the bristles are inclined forward (only

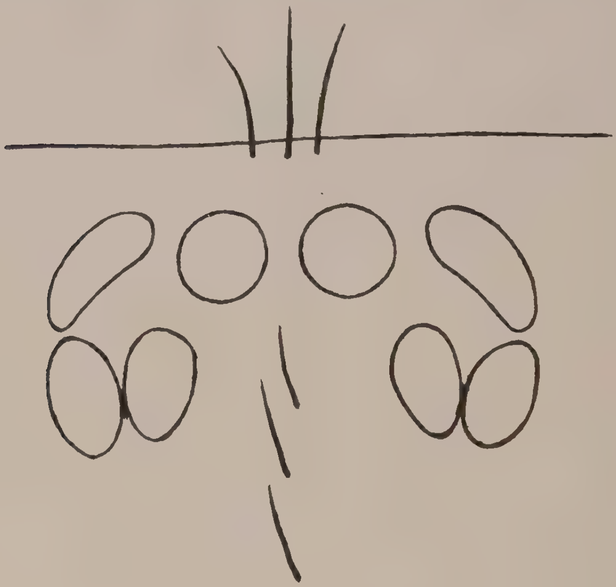

FIGURE 5, *Phaeoclita fauna* Simon. Eyegroup of female.

three of these bristles are shown in our figure), whereas the bristles in the lateral rows are inclined obliquely inward and backward. *Cheliceræ* powerful, normal, covered in front with spine-like bristles. Rastellum composed of 5 heavy spines. Four of these form a row at the end of the basal joint, while the fifth and heaviest spine is situated behind the others on a level with and directly to the inside of the pro-articulation of the fang. The margins are very long. Promargin with six teeth of which the distal fifth tooth is the largest. This row of teeth is continued proximally by a row of short black spines which at the first glance resemble teeth. Scopula on retromargin only. Prolateral surface covered with numerous bristles, retrolateral surface smooth. Fang rather short and evenly curved. *Lip* immobile, though clearly delimited at base. It is wider than long in ratio 13: 11, with parallel sides and rounded angles, truncated anteriorly and covered with a few brown bristles. *Maxillæ* with inner angles produced at both ends, the proximal angle with eight cuspules. Scopula well developed, occupying entire inner edge except the distal inner lobe. Surface clothed with short brown bristles. Insertion of palp terminal. *Sternum* longer than wide in ratio 43/28, oval, wider behind than in front, narrowly produced between the fourth coxæ, clothed with brown bristles. First coxæ wide apart, almost as much as second coxæ. Posterior sigilla circular, submarginal. Anterior legs slender, posterior legs, especially their femora, heavier. Third leg (Figure 6) very spinose, the others not nearly so. First tarsus very lightly scopulate to base, the other tarsi setose. Claws three. Upper claws with a double series of teeth, 10-11 on first and second tarsi, 4 on third and fourth tarsi. Third claw smooth. Ungual tufts wanting. Palpal claw with a pro-row of 8 teeth. Trichobothria very long, in two rows on all tarsi, less regularly arranged on metatarsi and tibiæ. *Legs* 4123.

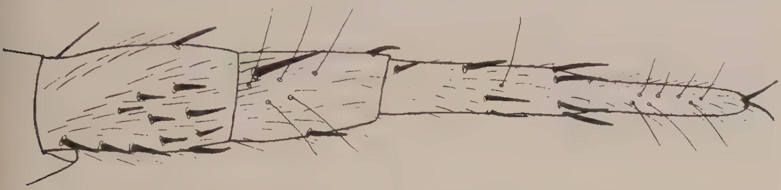

FIGURE 6, *Phaeoclita fauna* Simon. Third left leg of female, dorsal view.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	2.050	1.312	1.558	1.230	0.820	6.970
II	1.271	1.189	1.189	1.025	0.779	5.453
III	1.271	0.820	0.615	0.820	0.615	4.141
IV	1.968	1.271	1.886	1.435	0.820	7.380
Palp	1.312	0.779	0.820	0.820		3.731

Spines. First leg. Femur—dorsal 1-1-1-1-1-1-1 bristles, prolateral 0, retrolateral 0, ventral irregularly distributed bristles all the way from base to end. Patella—ventral 1-1 heavy curved bristles, elsewhere irregularly distributed bristles of various length. Tibia—dorsal 1-1 very long erect bristles, the first of which is near base, the second in middle. Prolateral 1-1-1-1 bristles, retrolateral 0, ventral 1-1-1-1. Metatarsus—dorsal 0, prolateral 0, retrolateral 1 at end, ventral 2-1r-2. *Second leg* same as first, except that the bristles are more slender. *Third leg.* Femur—dorsal 1 bristle near base, prolateral 0, retrolateral 0, ventral a prorow of bristles in distal half. Patella—dorsal 12 spines and several bristles arranged so that the oblique bald stripe separates 11 pro-spines from a single retrospine as shown in Figure 6. The disposition of these spines is such as to make their terminology impossible. No spines elsewhere on patella. Tibia—dorsal 1 at base, prolateral 1 in middle, retrolateral 1 at end, ventral 1p-2 stiff bristles. Metatarsus—dorsal 1-2, prolateral 1-1, retrolateral 1-1-1, ventral 1p-2. *Fourth leg* same as third, except: Femur—dorsal a fringe of 9 or 10 apical spines. Patella—0. Tibia—prolateral 0, retrolateral 1-1 slender spines, ventral 1p-2-2 stiff bristles. Metatarsus—dorsal 0, prolateral 0, retrolateral 1-1, ventral 1p-1p-1p-2. *Palp.* Femur—dorsal 1-1-1 bristles near end, ventral two irregular rows of bristles, elsewhere 0. Patella—dorsal 1-1 bristles, the first at base, the second before middle. Prolateral 1-1, elsewhere 0. Tibia—dorsal 0, prolateral 1-1-1-1 slender spines, retrolateral 0, ventral 2-2-4. Terminal joint—ventral 2-2, elsewhere 0. Terminal joint lightly scopulate to base. *Abdomen* 3.608 mm. long, 2.050 mm. wide, sparsely clothed with rust-colored hair. Second pair of spinnerets three-jointed, median joint shortest, terminal joint cone-shaped, shorter than basal joint. *Color in alcohol:* Above, carapace and cheliceræ orange, legs yellow, abdomen purplish-grey, spinnerets light yellow. A narrow, black, not clearly defined marginal line around the carapace. Below, light yellow, legs slightly darker. Field note of Dr. Shoemaker: "From burrow about 5 or 6 inches deep in gravelly clay near shore. East side St. Thomas Harbor. July 1915."

SUB-ORDER ARACHNOMORPHÆ

Family Uloboridæ

Miagrammopes ciliatus sp. nov. (Figure 7).

Female. Total length with cheliceræ 4.1 mm. Carapace 1.147 mm. long, flat, peculiar in shape. Its posterior two-thirds have parallel sides and the width of the carapace here is 0.703 mm. One-third from anterior end the edge of the carapace suddenly bulges out, forming on each side a tubercle for the PLE, so that the width of the carapace here is 0.740 mm. From the PLE anteriorly the carapace narrows down to the width of the cheliceræ and presents a distinct angle with the PLE for apex. Eyes 4, in a slightly recurved row, anterior eyes entirely wanting. Ratio of eyes AME : ALE : PME : PLE = 0 : 0 : 2 : 2. PME separated from each other by almost five diameters, from PLE by three diameters and from edge of clypeus by three diameters. The carapace is sparsely clothed with very fine hair. *Cheliceræ* stout, but short, with a darker colored boss, which however has no definite limits. Margins oblique, apparently smooth, but since the cheliceræ are shorter than the maxillæ it is difficult to be quite certain in this respect. Fang weak. *Lip* pointed, considerably longer than wide, reaching beyond middle of maxillæ. *Maxillæ* long, parallel, with short and scanty scopula but long serrula. *Sternum* with indefinite lateral limits, but evidently much longer than wide, narrowly produced between the anterior two pairs of coxæ. Second pair of coxæ separated by a considerable space from the third pair. First legs heavy and long, second and third

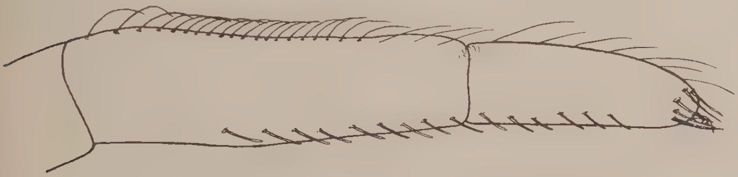

Figure 7, **Miagrammopes ciliatus** sp. nov. Fourth right leg of female.

pair slender and short, fourth pair long and heavy, but not as long nor as heavy as first. No spines and little hair on legs. Calamistrum extends over 2/3 of the full length of the fourth metatarsus. A row of peculiar short, blunt spines on retroventral surface of fourth metatarsus and tarsus (Figure 7). A retrolateral row of 4 or 5 cilia on second femur in proximal half. A similar prolateral row on third femur and a few irregularly situated cilia on prolateral surface of fourth femur. Claws concealed by spurious claws. *Legs* 1423.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	1.406	1.369	0.888	0.444	4.107
II	0.777	0.592	0.370	0.296	2.035
III	0.518	0.518	0.333	0.333	1.702
IV	0.999	1.332	0.518	0.296	3.145

Palpi very small. *Abdomen* 2.665 mm. long, 0.738 mm. wide, with a dorsal conical elevation above the spinnerets. Anal tubercle long, two jointed. Spinnerets 6. Anterior pair heavier and slightly longer than posterior pair. Cribellum entire. *Color* generally olivaceous. Sides of abdomen with irregular white spots. Venter of abdomen with a pair of longitudinal, parallel dark lines extending from genital fold almost to cribellum. July 1915. A single female from St. Thomas in the collection of Dr. Shoemaker.

FAMILY DYSDERIDÆ

Ariadna arthuri sp. nov. (Figures 8, 9 and 10).

Female. Apparently immature, but probably just before the last moult. Total length 6.24 mm. Carapace 2.665 mm. long, 1.640 mm. wide in middle, 1.107 mm. wide in front. Thoracic groove wanting. Head very long, well marked by the sulci and the bristles which form parallel longitudinal rows on the head, while on the thorax the rows are oblique, less perfect, converging on the spot where the thoracic groove would be if it were present. Anterior portion of head with parallel sides. Generally speaking, thoracic portion also with parallel sides, but there is an angular protrusion opposite the second pair of coxæ. Anterior edge of head strongly recurved and eyegroup much narrower than head, being only 0.629 mm. wide. Eyes 6, AME wanting. PME (central pair) form a strongly recurved line with the ALE and a gently recurved line with the PLE. Ratio of eyes AME: ALE: PME: PLE = 0: 4: 3.5: 4. PME subcontiguous, separated only by a black line. PME separated from ALE by 2.5/3.5 of their diameter, from PLE by 3/3.5 of their diameter. Laterals contiguous. Clypeus equal to diameter of laterals. Distance of PME from edge of clypeus equal to twice their diameter. Edge of carapace forms anteriorly on each side a sharp, dark brown lobe which has apparently something to do with the peculiar outer angle of cheliceræ (Figure 8). Carapace moderately high, highest at end of cephalic portion, *i.e.* about 2/3 from anterior end. From this point the carapace slopes gently forward and more rapidly backward. *Chelicerœ* short, cone-shaped, barely reaching end of maxillæ, with an outer, sharp, black, cone-shape projection near base (Figure 8), corresponding to the angular lobe of the

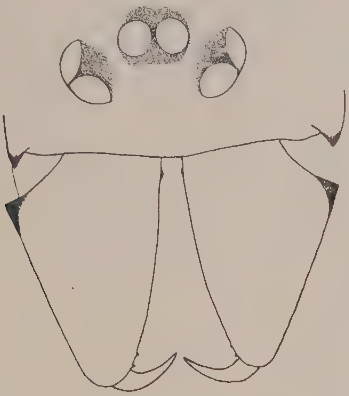

FIGURE 8, **Ariadna arthuri** sp. nov. Cheliceræ, lip and maxillæ of female.

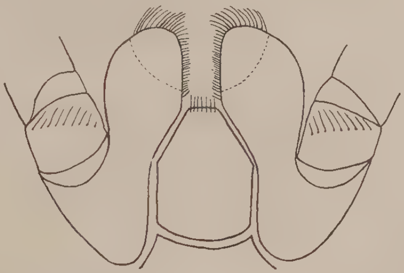

FIGURE 9, **Ariadna arthuri** sp. nov. Lip and maxillæ of female.

carapace edge. Margins extremely short, forming together a parabolic curve at the vertex of which a minute tooth is situated. A similar minute tooth at each end of the parabola, immediately under the fang. Scopulæ wanting, but numerous hairs on prolateral surface of basal joint. *Lip* much longer than wide in ratio 14 : 10, considerably surpassing middle of maxillæ. Base of lip strongly procurved, sides parallel in proximal half, then converging. Anterior end of lip narrowly truncated (Figure 9).

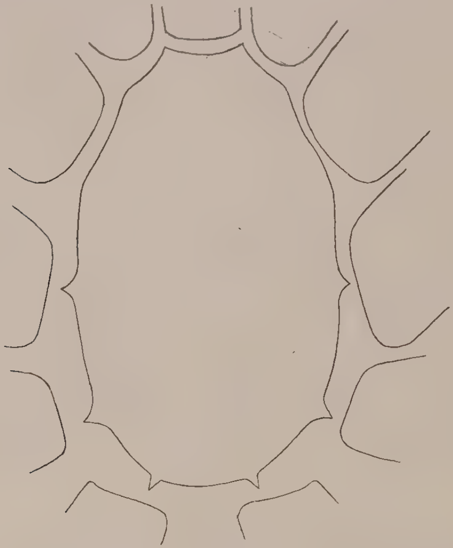

FIGURE 10, **Ariadna arthuri** sp. nov. Sternum of female.

Maxillæ long, slightly converging, almost without truncature, but entire tip white as indicated by a dotted line in Figure 9. Scopula reaching the level of the lip. Serrula very long and dark, extending almost to the trochanter. *Sternum* longer than wide in ratio 45 : 27, narrowed in front to width of lip. Lateral margins with spine-like projections opposite second, third and fourth pair of coxæ. (Figure 10.) Fourth coxæ subcontiguous. *Legs* 1243.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	1.924	2.516	1.480	0.629	6.549
II	1.813	2.405	1.369	0.629	6.216
III	1.480	1.739	1.036	0.555	4.810
IV	1.850	2.405	1.110	0.629	5.994
Palp	0.814	0.814	0.629		2.331

Third pair of legs directed forward. First and second femur and first and second metatarsus distinctly curved dorsoventrally. First femur is, in addition, curved laterally in the fashion of the mathematical symbol ∼, and has a very large spine on the prolateral convex curvature, directed ventrally at right angles to longitudinal axis of femur.

Spines on legs. First leg. Femur—dorsal 0-1 near end, prolateral 1-1-1, first spine directed ventrally and very large, retrolateral 0-1 near end, ventral 0. Patella—0. Tibia—dorsal 0, prolateral—1-1-1 short spines, retrolateral 1-1-1 short spines, shorter than prolateral spines, ventral 2-2-2-2-2-2-2, of which the first, third and fifth pair are much longer than the others. Metatarsus—ventral 2-2-2-2-2-2-2, of which the first and the fourth spines are longest, then the third and the seventh. Elsewhere 0. *Second leg* same as first, except: Femur—prolateral 0-0-1. Tibia—retrolateral 1-1 on the right leg and 0-1-0 on the left leg, in both cases short spines. *Third leg.* Femur—dorsal 0, prolateral 0-0-1 very short, slender spine, retrolateral 0-0-1 very short, slender spine, ventral 0. Patella—0. Tibia—ventral 1-1, elsewhere 0. Metatarsus—dorsal 0, prolateral 1-1-1, retrolateral 0, ventral 1r-1r-2. *Fourth leg* has no spines at all. *Palp* has stout spines on the following joints: Tibia—prolateral 2-3, Terminal joint—prolateral 3-IV-2-IV. There are therefore 12 spines only on each palp. Claws 3. Upper claws with 5 teeth, third claw smooth. Palpal claw short, smooth, attached to end of terminal joint by almost its entire length, so that only the tip of the claw is free. Trichobothria wanting. *Abdomen* clothed with simple hair. *Color* in alcohol: legs and carapace rufous, abdomen dark grey, below same as above. One female in my collection, taken from under the bark of a log, in Sta. Maria Bay, July 28. Named in honor of Mr. Arthur Lindborg, son of Mr. A. E. Lindborg.

Family Theridiidæ

Spintharus minutus sp. nov. (Figure 11).

Female. Total length 2.46 mm. Carapace 0.962 mm. long, 0.814 mm., wide, flat, oval, narrowed toward the front. Thoracic depression one-third from posterior edge. Anterior row of eyes recurved, posterior row straight. Laterals contiguous. Anterior eyes almost equidistant, AME a little closer together than to ALE. AME slightly smaller than the other three pairs. PME wider apart than from PLE. Quadrangle wider behind than in front in ratio 6.5 : 5, and wider than long in ratio 6.5 : 5. Clypeus 5/5 the length of the quadrangle. Surface of carapace devoid of hair. *Sternum* almost triangular, about as wide as long, pointed between hind coxæ which are separated from each other by their width. *Legs* 1423.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	1.517	1.480	1.295	0.518	4.810
II	1.110	1.016	0.740	0.370	3.236
III	0.777	0.629	0.518	0.333	2.257
IV	1.110	1.050	0.777	0.407	3.344

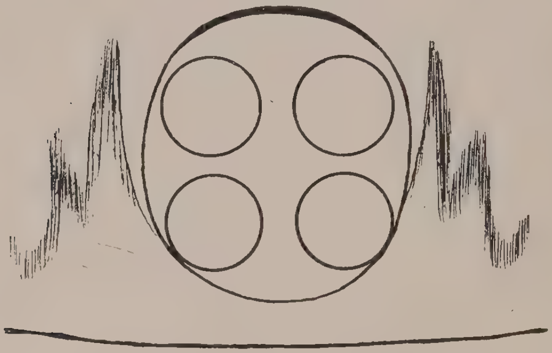

FIGURE 11, **Spintharus minutus** sp. nov. Epigynum.

Legs clothed with numerous bristles of the same color and appearance as the spines, so that the latter cannot be distinguished with any degree of certainty. Certain, however, is the presence of a middorsal spine on all patellæ and tibiæ. Trichobothria seem to be completely wanting. Comb composed of seven typically serrated bristles. Upper claws with but one minute tooth. Third claw very long, thin and curved S-like. Palpal claw smooth, almost straight. *Abdomen* clothed sparsely with erect bristles. Epigynum (Figure 11) with four round receptacles. *Color in alcohol* generally very pale yellow, sternum almost white. A prolateral black round spot on all patellæ and a similar prolateral and retrolateral spot on all tibiæ near end. Two minute black spots also at end of all metatarsi. One female from St. Thomas in the collection of Dr. Shoemaker, taken July 1915.

FAMILY ARGIOPIDÆ

Micrathena militaris (Fabricius) (Figures 12 to 15).

M. militaris Reimoser. Verh. k. k. zool. bot. Ges. Wien, 1917, p. 126.

Reimoser gives a place to this species in his analytical key, even though he gives no description of it and merely mentions the brief descriptions of Fabricius, Olivier and Walckenaer.

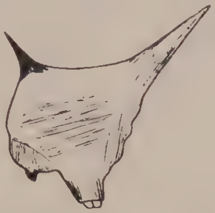

FIGURE 12, *Micrathena militaris* (F). Abdomen of female viewed from the left side.

Olivier states that the species occurs in Cayenne and Surinam, which is not impossible; but, as in the case of many old records, this statement must be taken with caution. The specimen described here fully conforms with the old descriptions and may be therefore regarded as certainly belonging to the species *M. militaris* (F.).

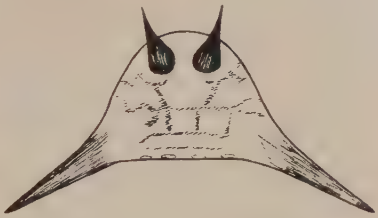

FIGURE 13, *Micrathena militaris* (F). Abdomen of female viewed from above.

Female. Total length in midventral line from end of cheliceræ to end of abdomen 8.16 mm. Carapace 2.880 mm. long, 2.496 mm. wide, glabrous. Thoracic depression transverse. Head clearly elevated above carapace, wide in front. Anterior margin truncated. Quadrangle on an elevation and laterals on each side on a distinct elevation also. First row of eyes shorter than second. Laterals separated from each other by more than their diameter (4.5/3.25 of their diameter). Quadrangle wider behind than in front in ratio 12:10, as long as wide behind. Ratio of eyes AME : ALE : PME : PLE = 4.25 : 3.25 : 5 : 3.25. AME separated from each other by 3/4.25 of their diameter, from ALE by 13/4.25 of their diameter, *i.e.* by more than three diameters. Clypeus equal to diameter of AME. Viewed from in front first row of eyes curved downward so gently that a straight line tangent to lower edge of AME cuts ALE 1/3 their diameter from upper edge. Second row, when viewed from in front strongly curved downward and PLE not visible in this position. Viewed from above with carapace horizontal, both rows appear slightly recurved. *Abdomen* in middorsal line 5.184 mm. long, in midventral line 4.992 mm. long. Width of abdomen between the bases of the posterior spines 5.760 mm. Span of posterior spines from point to point 13.4 mm. These posterior spines are brown, strongly divergent, stout, pointed, merging at base with the surface of the abdomen from which they may be distinguished only by their color. The two dorsal spines are black, much smaller, directed upward and slightly forward, diverging so little from each other that they are almost parallel. They are very stout at base, but very soon become slender. The spinnerets are on a long

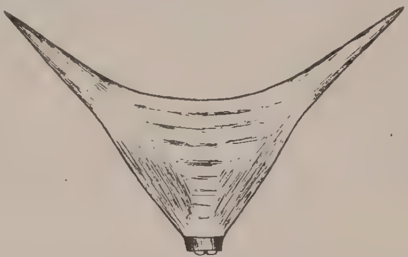

FIGURE 14, *Micrathena militaris* (F). Abdomen of female viewed from behind.

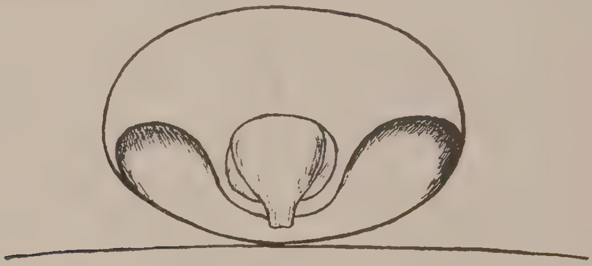

FIGURE 15, *Micrathena militaris* (F). Epigynum.

tubular turret which is slightly farther from anterior than from posterior end of abdomen. *Legs* 4123, without spines, femora covered with numerous tubercles. Epigynum shown in Figure 15. Two females collected by Dr. Shoemaker in St. Thomas.

FAMILY SELENOPIDÆ

Selenops lindborgi sp. nov. (Figure 16).

Female. Total length with cheliceræ 12.3 mm. Carapace 4.2875 mm. long, 4.550 mm. wide between second and third pair of legs, 2.3625 mm. wide in front, very flat. Thoracic groove longitudinal, not clearly distinct. Sulci broad and clearly defined. Lateral margins dark and fringed with stiff, brown setæ. Surface of carapace rufous, sparsely clothed with short, dark hair. White hair behind eyegroup and on clypeus. Arrangement of eyes typical for genus. Width of eyegroup 2.5375 mm. Ratio of eyes AME : ALE : PME : PLE = 7 : 10 : 3.5 : 11. AME separated from each other by their radius, from ALE by half their radius. ALE separated from PLE by 13/10 of their diameter, from the small PME by 6/10 of their diameter. PME separated from PLE by 10/11 of the diameter of the latter. PLE separated from each other by 2.035 mm. All eyes round, but PME alone iridescent white. Viewed from above PME are not visible and the six visible eyes form an evenly recurved line. Viewed from in front, when upper edge of ALE is in a straight line with upper edge of PLE, the four anterior eyes are curved upward and the PME appear in a position in which a straight line tangent to the lower edge of the ALE is also tangent to the upper edge of the PME. Clypeus 5/7 diameter of AME, or equal to radius of ALE. Margin of clypeus fringed with a row of stiff bristles. One long, median bristle between AME. *Cheliceræ* distinctly geniculated, clothed with black bristles and white hair. Promargin with 3 teeth of which median is strongest and nearer to proximal than to distal tooth. Retromargin with 2 teeth which are very far apart, the proximal tooth being almost opposite the median promarginal tooth, the distal tooth at the edge of the semilunar articulation membrane. Scopula on promargin only. Boss very prominent, light yellow. *Lip* wider than long in ratio 22 : 20, with deep lateral excavations in proximal third, where the lip is articulated to the maxillæ close to their base. Anteriorly lip is rounded and fringed with long, converging dark bristles. Lip does not reach middle of maxillæ. *Maxillæ* parallel. Scopula extends over truncature to the level of the lip. Serrula marginal, rough, comparatively short, just reaching outer rounded angle. *Sternum* flat, widely truncated in front, bifid behind. First coxæ very far apart, fourth coxæ separated from each other by 30/25 of their maximum width. Fourth coxæ a little longer than the other three pairs. Sternum slightly wider than long in ratio 62:59. *Legs* laterigrade, 2341.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	4.592	6.150	3.526	1.640	15.908
II	5.125	6.355	3.567	1.640	16.687
III	5.125	5.658	3.708	1.640	16.131
IV	5.330	5.412	3.608	1.640	15.990
Palp	1.184	1.435		1.312	3.931

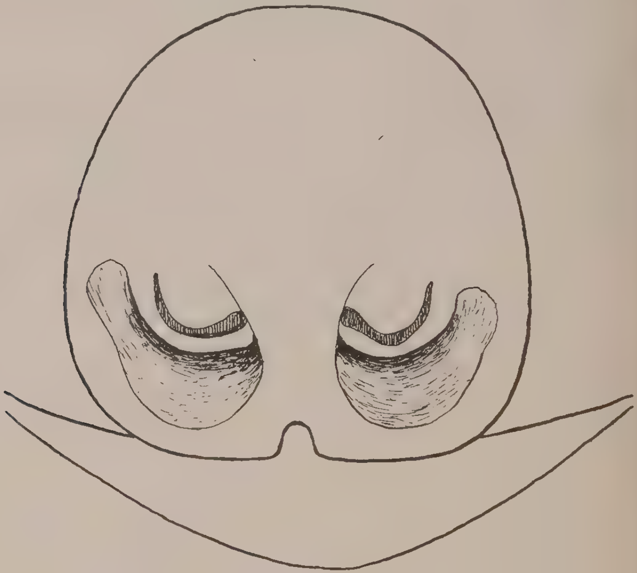

Figure 16, **Selenops lindborgi** sp. nov. Epigynum.

Spines. First leg. Femur—dorsal 1-1-1, prolateral 0-1-0, in middle, elsewhere 0. Patella—dorsal 0-1 bristle, elsewhere 0. Tibia—ventral 2-2-2 very long spines, elsewhere 0. Metatarsus ventral 2-2, elsewhere 0. *Second leg* same as first, except: Femur—prolateral 1-1-0. *Third leg.* Femur—dorsal 1-1-1, prolateral 1-1-0, elsewhere 0. Patella—dorsal 1-1 bristles, elsewhere

0. Tibia—ventral 2-2, elsewhere 0. Metatarsus—ventral 2-2, elsewhere 0. *Fourth leg* same as third, except: Metatarsus—ventral 2-1p-1p, the apical spine very small. *Palp.* Femur—dorsal 1-2, prolateral 0-1, retrolateral 0-1, ventral 0. Patella—dorsal 1-1 stiff bristles, prolateral 1, elsewhere 0. Tibia—dorsal 1-1, prolateral 2-1, retrolateral 1, ventral 0. Terminal joint—12 spines the arrangement of which is difficult to ascertain. First and second pair of tarsi lightly scopulate to base, third and fourth pair in apical third. No scopulæ on other joints of legs. Claw tufts well developed. Claws 2, smooth. Palpal claw with 6 teeth. Epigynum shown in Figure 16; with two deep pockets. Opening of uterus very wide. *Color in alcohol:* Carapace rufous with narrow, dark lateral marginal line. Cheliceræ brown with yellow boss. Palpi yellow. Legs yellow banded with brown. Lip and maxillæ rufous. Sternum, ventral surface of abdomen and of legs of about the same light yellowish-grey color. Above, abdomen almost grey, with a suggestion of brown. Sides and posterior end of abdomen brown. A characteristic feature of the coloration is the festoon-shaped posterior edge of the dorsum. One female and one young specimen found under the bark of a log in Sta. Maria Bay, St. Thomas, July 28. Named in honor of Mr. Arthur E. Lindborg.

FAMILY CLUBIONIDÆ

Eutychuroides gen. nov.

Closely allied to *Eutychurus* and *Strotarchus.* Has the spinnerets and the eyes of the former, but the fourth legs are longer than the first pair, carapace has a longitudinal groove and retromargin of cheliceræ has only two teeth. Type **E. fuscus.**

Eutychuroides fuscus sp. nov. (Figure 17).

Female. Total length with cheliceræ, but without spinnerets, 14.8 mm. Carapace 5.568 mm. long, 3.840 mm. wide, gradually narrowed toward the front where it measures 2.688 mm. Head not clearly set off from rest of carapace. Thoracic groove longitudinal, deep and long, its middle situated exactly one quarter from posterior edge. Carapace low, clothed with fine, short hair, directed inward and forward. The space at posterior end of carapace, corresponding in size to anterior surface of abdomen completely devoid of hair. Median strip behind the eyegroup as far as thoracic groove sparsely clothed with long, stiff hair. Eyegroup much narrower than width of carapace in eye-region. Viewed from above, first row of eyes is very slightly recurved, second row as slightly procurved and somewhat longer. All eyes round, diurnal. Ratio of eyes AME : ALE : PME : PLE = 6 : 5 : 5 : 5. AME separated from each other by 4/6 of their diameter, from ALE by 3/6 of their diameter. PME separated from each other by 7/5 of their diameter, from PLE by 8/5 of their diameter. Laterals separated by 4/5 of their diameter. Quadrangle almost as wide in front as behind, the ratio being 16 : 16.5, almost as wide behind as long in ratio 16.5 : 17. Clypeus equal to diameter of AME. Edge of clypeus with a row of stiff bristles. *Chelicerœ* powerful, geniculated, with well-developed boss, anteriorly clothed with bristles. Margins oblique. Promargin with three teeth of which median is strongest. Retromargin with two small teeth separated from each other by the width of their base. Scopula on promargin only. *Lip* free, longer than wide in middle in ratio 26 : 24. Lateral excavations in basal third deep. From here on the sides of the lip converge in a curve toward end which is distinctly emarginate, strongly rebordered and clothed with stiff hair. End of lip surpasses middle of maxillæ. *Maxillœ* without oblique impression, with outer angle of base reaching almost to end of lip, so that trochanter appears to be inserted in middle of maxillæ. Outer edge rounded from scopula to outside, then almost straight with just perceptible concavity. Truncature short, with yellowish-brown scopula ending in front with a tuft of curved brown hair. Serrula marginal, rather short reaching to outer angle. *Sternum* oval, flat, longer than wide in ratio 35 : 27, truncated in front to width of lip, pointed behind, clothed with very short fine, recumbent and with long, stiff, erect hair. Coxæ all of the same type, fourth pair contiguous or nearly so. *Legs* 4123.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	3.456	4.992	2.304	1.632	12.384
II	3.360	4.896	2.304	1.632	12.192
III	3.456	3.840	2.400	1.632	11.328
IV	4.128	5.376	4.128	2.016	15.648
Palp	1.632	1.632		1.440	4.704

Third trochanters slightly, fourth trochanters strongly notched. All tarsi scopulate to base, scopulæ of first and second pair quite heavy, those of fourth pair alone divided longitudinally by two rows of spine-like bristles. First and second metatarsi heavily scopulate to base, scopula extending even to end of tibia. Third metatarsus lightly scopulate to base, scopula divided by a single row of long setæ. Fourth metatarsus lightly scopulate at end, scopula imperceptibly merging with the rather heavy hair on sides of segment. Terminal joint of palp lightly scopulate at end. Ungual tufts well developed. Claws two, similar, strongly bent, with five teeth each. Palpal claw of the same type, but less powerful. Trichobothria difficult to see, apparently only on tarsi, in a single row. Hair of two kinds, long stiff bristle-like, inclined at an angle of about 30° and shorter, less numerous, erect, stiff hair.

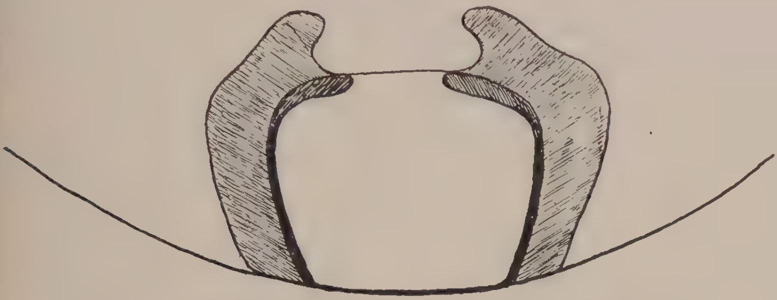

FIGURE 17, **Eutychuroides fuscus** gen. et sp. nov. Epigynum.

Spines. First leg. Femur—dorsal 1-0, prolateral 0-1, elsewhere 0. Patella—dorsal 1 bristle at end, elsewhere 0. Tibia—dorsal 1r-1 fine bristles, ventral 2-2-0 very fine spines recognizable as such only by the darker spot at base, elsewhere 0. Metatarsus—0. *Second leg* same as first, except: Femur—dorsal 1-1, prolateral 0-2. Patella—dorsal 1-1 bristles. Tibia—ventral 1r-1r distinct spines. Metatarsus—ventral 1-0. *Third leg.* Femur—dorsal 1-1-1, prolateral 1-1-1, elsewhere 0. Patella—dorsal 1-1 bristles, elsewhere 0. Tibia—dorsal 0-1r-0, prolateral 1v-1d-1v, retrolateral 1-1, ventral 2-2-2. Metatarsus—dorsal 1r-2-2, prolateral 1-1-0, retrolateral 1-1, ventral 2-0-2, the first pair very long, the apical pair quite short. *Fourth leg* same as third, except: Femur—retrolateral 0-0-1, Tibia—dorsal 1-1, prolateral

1-1, Metatarsus—dorsal 2-2-2, prolateral 1-1-1, retrolateral 1-1-1, ventral 2-2-2. *Palp.* Femur—dorsal 1-1-1, prolateral 0-0-1, elsewhere 0. Patella—dorsal 1-1, prolateral 1, elsewhere 0. Tibia—dorsal 1-1, prolateral 2-1, retrolateral 1, ventral 0. Terminal joint—dorsal 1-0, prolateral 1-1, retrolateral 0, ventral 0-2 near end.

Ratio of first patella to first tibia = 27 : 34, of fourth patella to fourth tibia = 26 : 37. Tracheal spiracle close to spinnerets. Colulus wanting. Posterior spinnerets long, their second joint longer than the basal joint. Abdomen clothed with fine recumbent hair. Epigynum shown in Figure 17. *Color in alcohol* light brown with spinnerets light yellow. Two females and one immature specimen collected by Dr. Shoemaker in St. Thomas.

Corinna cleonei sp. nov. (Figures 18 and 19).

Female. Total length with cheliceræ 11.5 mm. Carapace 4.28 mm. long, 3.50 mm. wide between second and third pair of coxæ, 2.71 mm. wide behind eyegroup. Thoracic groove longitudinal. Head convex, broad, short in proportion to its width, the ratio of length to width being only 33 : 31. First row of eyes 1.406 mm. wide, second row 1.729 mm. Viewed from above, first row is gently recurved, second still more gently procurved, almost straight. Ratio of eyes AME : ALE : PME : PLE = 6.5 : 5.75 : 4.5 : 4.5. AME separated from each other by 4.5/6.5 of their diameter, from ALE by 5/6.5 of their diameter. PME separated from each other by 9/4.5 of their diameter, from PLE by 11/4.5 of their diameter. ALE separated from PLE by 3.5/4.5 of the diameter of the latter. Quadrangle as wide behind as in front (17/17), slightly wider than long in ratio 17 : 16. Clypeus equal to diameter of AME. Carapace covered with short black spines. Edge fringed with quite short curved spines, except at postero-lateral angles where there are 5 longer, curved spines. Surface of carapace roughly granular. *Chelicerœ* powerful, gibbous, with strong boss. Promargin with three teeth of which median is largest. Retromargin with five teeth of which the proximal one is distinctly larger than the other four which are subequal. A thick scopula of stiff bristles on promargin only. *Lip* rectangular, wider than long in ratio 22 : 20. At base of lip are lateral projections which correspond to angular projections of the anterior margin of the sternum (Figure 18). In the above ratio the width of the lip is measured in middle. Measured at base, the ratio is

24:20. The angular projections of the lip are darker than the rest and the base of the lip is distinctly rebordered. The surface of the lip is glabrous. Stiff black bristles fringe the squarely truncated anterior end. The anterior angles are rounded. The lip does not reach middle of maxillæ. *Maxillæ* parallel to each other and with straight inner and outer edges. Truncature short, white, covered with a thick scopula forming a straight line not extending over inner edge. Surface of maxillæ glabrous, very sparsely covered with stiff black hairs. Serrula very short and visible only from in front. *Sternum* rebordered (Figure 18), longer than wide in ratio 54:52, widely truncated in front and drawn out into anterolateral, short, angular projections corresponding to the basal projections of the lip. Surface of sternum flat, granular, sparsely clothed with long, stiff, erect and shorter, fine, recumbent hair. First pair of coxæ wide apart, fourth pair contiguous. *Legs* 4123.

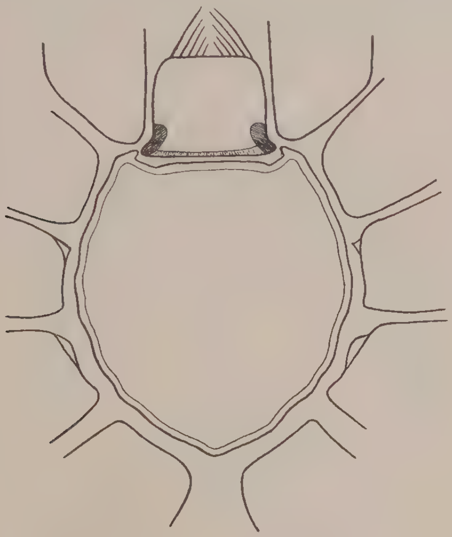

FIGURE 18, **Corinna cleonei** sp. nov. Sternum and lip of female.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	3.325	4.375	2.450	1.575	11.725
II	2.975	3.850	2.015	1.400	10.240
III	2.450	3.325	2.100	1.225	9.100
IV	3.590	4.635	3.235	1.485	12.945
Palp	1.575	1.575		1.400	4.550

Spines. First leg. Femur—dorsal 1-0-1, prolateral 0-1-1, elsewhere 0. Patella 0. Tibia—ventral 2-2-2-2, elsewhere 0. Metatarsus—ventral 2-2, elsewhere 0. *Second leg* same as first, except: Femur—prolateral 1-1-1, Tibia—ventral on left leg 2-1r-1r-2, on right leg 1r-1r-2-2. *Third leg.* Femur—dorsal 1-1-1, prolateral 1-1-1, retrolateral 0-1-1, ventral 0. Patella 0. Tibia—dorsal 0, prolateral 1-1, retrolateral 1-1, ventral 2-2-2. Metatarsus—dorsal 0, prolateral 1 in middle, retrolateral 1 in middle, ventral 2-2-2. *Fourth leg* same as third, except: Femur—prolateral 0-0-1, retrolateral 0-0-1. Tibia—ventral first and second retrospines on left leg very small, on right leg second retrospine wanting. *Palp.* Femur—dorsal 0-1-1, prolateral 0-0-1, elsewhere 0. Patella—prolateral 1, elsewhere 0. Tibia—dorsal 1 in middle, prolateral 1-1-1, elsewhere 0. Terminal joint—prolateral 2-0, elsewhere 0. All spines short. Trichobothria in one row on tarsi and metatarsi, in two rows on tibiæ. Legs clothed with short spinelike hair and fine recumbent hair. A few erect fine hairs also present. A dorsal longitudinal median line free of all hair on all tarsi and metatarsi and a pair of similar lines on all tibiae. True scopulæ wanting, but spinelike hair on all tarsi much more numerous, simulating scopulæ. Ungual tufts well developed. Claws 2, concealed by the ungual tufts, similar with two minute teeth. Palpal

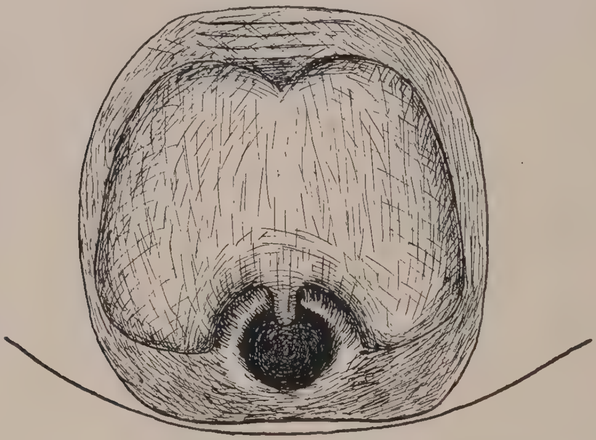

FIGURE 19, **Corinna cleonei** sp. nov. Epigynum.

claw with 5 teeth. *Abdomen* clothed with short, curved, dark, spinelike hair, and longer thin, recumbent hair of lighter color. Two pairs of round, chitinized spots on back of abdomen in middle, posterior pair wider apart than the anterior pair. Spinnerets about equal in length, medians a little shorter. Anterior pair of spinnerets contiguous. Second joint in both anterior and posterior spinnerets hemispherical. Two large spigots on second joint of posterior spinnerets and three spigots on median spinnerets. Colulus replaced by a tuft of hair. Epigynum as shown in Figure 19 rufous, with a circular vulva near posterior edge, overhung by a short median tongue. Below the vulva the epigynal plate is bent at right angles, forming a narrow rectangular plate above genital opening, not shown in the figure because it is visible only when examined from behind. *Color in alcohol:* Carapace mahogany-red, cheliceræ mahogany-black, legs rufous, abdomen grey-brown. Below, cheliceræ mahogany-red, legs rufous. Abdomen grey-brown, but of lighter shade. Spinnerets yellow. This species is closely related to *C. saga* F. Cambridge, from which it may be differentiated by its larger size, its epigynum and the arrangement of spines on tibiæ. One female from Sta. Maria Bay, taken from under the bark of a log on July 28. Named in honor of Miss Cleone Lindborg.

Syrisca hirsuta Petrunkevitch (Figures 20 and 21).

A male of this species, described by me from Panama, was taken from a small tube-web in a crevice of a rock in Sta. Maria Bay, St. Thomas, July 28. It is slightly larger than the type and the ratio of its eyes is different. Nevertheless I think it belongs to the same species, as it has the same appearance, the same color and same type of pattern, also the same structure of the palp. In view, however, of the slight differences and the possibility of the existence of a sub-species or local race, I give here a detailed description of the specimen from St. Thomas.

Male. Total length with cheliceræ and spinnerets 8.64 mm. Carapace 3.075 mm. long, 2.130 mm. wide between the second and third pair of legs, 1.184 mm. wide in the region of the posterior eyes. Abdomen 3.690 mm. long without spinnerets, 1.960 mm. wide in middle. Carapace low. Thoracic groove longitudinal, deep. Eyegroup 0.740 mm. wide; first row of eyes 0.645 mm. wide, recurved; second row very gently procurved, almost straight. Viewed from in front first row straight, second row curved downward. Ratio of eyes AME : ALE : PME : PLE = 5 : 3.5 : 3 : 3.5. AME separated from each other by 1.5/5 of their diameter, from ALE by 1.25/5 of their diameter. PME separated from each other by 3/3 of their diameter, from PLE by the same distance. Laterals separated from each other by 1.5/3.5 of their diameter. Quadrangle as long as wide, and as wide behind as in front. Clypeus equal to 3/5 of the diameter of AME. Edge of clypeus with 6 long bristles and a median, long, recurved bristle between AME, in line with their lower edge. Lateral margins of carapace fringed with long bristles directed forward. Median yellow band on carapace with a few irregularly arranged bristles extending to thoracic groove. Rest of carapace thickly clothed with recumbent black hair. *Chelicerœ* long, powerful, with strong boss. Dorsal surface covered with long bristles. Promargin with three teeth of which median is strongest. Retromargin with two teeth. Scopula on promargin only. *Lip* as long as wide, with deep lateral excavations at base, truncated and slightly emarginate at end, rebordered. Lip barely reaches middle of maxillæ. *Maxillœ* almost parallel to each other, outer edge slightly concave, outer angle rounded, oblique depression barely noticeable. Scopula extending down to level of lip. Truncatures short, forming between them a right angle. Serrula long and fine, reaching to middle of outer angle. *Sternum* flat, oval, longer than wide in ratio 43 : 35, truncated in front to considerably more than width of lip, pointed behind in front of fourth coxæ which are contiguous. First coxæ wide apart. Surface and especially edge of sternum covered with long, erect hair and fine, recumbent hair. *Legs* 4123.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	2.460	3.403	1.845	1.312	9.020
II	2.337	3.280	1.640	1.230	8.487
III	2.050	2.583	1.763	1.199	7.595
IV	2.870	3.895	3.116	1.640	11.521
Palp	1.025	1.166	0.738		2.929

Patella of all legs very long. For example, ratio of patella to tibia in first pair of legs is 25 : 52, *i.e.* almost half as long as tibia. All trochanters notched. All tarsi scopulate to base, those of fourth pair divided longitudinally by a band of setæ. First and second metatarsi scopulate almost to base, third and fourth without scopula. True ungual tufts wanting, but scopula projecting below claws. Claws two, similar, with five teeth. Trichobothria in two rows on tibiæ, metatarsi and tarsi. Legs rather hairy, with three kinds of hair: long, stiff hair inclined at an angle of about 45°, shorter, erect hair and fine, grey recumbent hair. Spines numerous and long, especially on posterior metatarsi. There is also a dorsal basal spine present on right fourth tarsus, but as it is wanting on the left tarsus and as there is no trace of a corresponding socket there, it is probable that the spine on the left tarsus represents merely a case of hyperdevelopment of a hair.

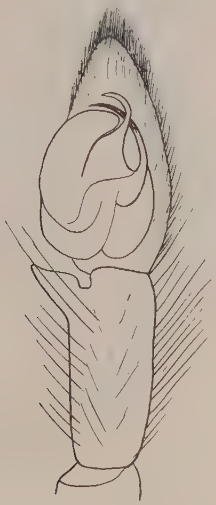

FIGURE 20, *Syrisca hirsuta* Petrunkevitch. Right palp of male from St. Thomas from below.

Spines. First leg. Femur—dorsal 1-1-0, prolateral 0-1-1 small spines near end, elsewhere 0. Patella—dorsal 1-1 bristles, elsewhere 0. Tibia—dorsal 1-0-1 bristles, ventral 2-2-2, elsewhere 0. Metatarsus—ventral 2-0 near base, elsewhere 0. *Second leg* same as first, except: Tibia—ventral 1r-2-2. *Third leg.* Femur—dorsal 1-1-1, prolateral 1-1-1, retrolateral 1-1-1, ventral 0. Patella—dorsal 1-1 bristles, elsewhere 0. Tibia—dorsal 1-1-1, the last one a bristle, prolateral 1-1, retrolateral 1-1, ventral 2-2-2. Metatarsus—dorsal 2-2, prolateral 1-1, retrolateral 1-1, ventral 2-0-2, apical pair small. *Fourth leg* same as third, except: Femur—retrolateral 0-1-1, Tibia—prolateral 1-1-1, Metatarsus—dorsal 2-2-2, ventral 2-2-2. *Palp.* Femur—dorsal 1-1-1, prolateral 0-2 at end, elsewhere 0.

Patella—dorsal 1-1 bristles, second bristle almost as strong as a spine, prolateral 1 bristle in middle, elsewhere 0. Tibia—dorsal 1 in middle, prolateral 2-2, elsewhere 0. Terminal joint—0. Tibia of palp with retrolateral apophysis of the same type as in the type specimen. Copulatory apparatus shown in Figure 20. As will be seen from a comparison of this figure with Figure 74 on page 151 in my paper on the Arachnida from Panama, the embolus in the St. Thomas specimen arises much higher and is in consequence much shorter. *Abdomen* (Figure 21) is fringed in front with dark, curved bristles and is clothed above and below with dark bristle-like hair and with fine, recumbent hair. The color of the abdomen is due, however, not to the color of the hair, but to pigmentation of the chitin itself of the abdominal wall. The pattern is the same as in the type specimen, *i.e.* consists of a yellow median band in anterior half, followed by several transverse yellow ribs or stripes, the rest of the surface being black. The ventral surface is considerably lighter, of a uniform yellowish-grey. The spinnerets are yellow, the posterior pair much longer than the others, two-jointed, the terminal joint long, cylindrical. The legs are of a greenish-

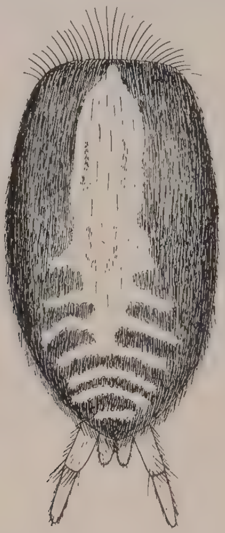

FIGURE 21, *Syrisca hirsuta* Petrunkevitch. Dorsal view of abdomen of male from St. Thomas.

grey color, first pair darkest. Cheliceræ, lip, maxillæ, sternum, palpi, coxæ and trochanters as well as venter in front of genital fold, yellow. The carapace is almost black with a yellow longitudinal median band of the width of the eyegroup, beginning immediately behind the latter and extending to posterior edge.

FAMILY LYCOSIDAE

Lycosa sancti thomasi sp. nov. (Figure 22).

Female. Total length 10.0 mm. Carapace 4.025 mm. long, 2.795 mm. wide between second and third pair of legs, 1.835 mm. wide in front. Thoracic groove longitudinal, well-developed, its anterior end two-thirds from front edge of carapace. Cephalothoracic and thoracic sulci all well developed. Sides of face rather steep. First row of eyes very gently curved downward. First row 0.925 mm. wide, second 1.050 mm., third 1.295 mm. Ratio of eyes AME : ALE : PME : PLE = 5.5 : 4 : 13 : 10. AME separated from each other by 3/3.5 of their diameter, from ALE by 3/3.5 of their diameter. ALE separated from PME by 4/4 of their diameter. PME separated from each other by 8/13 of their diameter, from PLE by 11/13 of their diameter. PLE separated from each other by 22/10 of their diameter. Quadrangle of posteriors wider than long in ratio 36 : 30, wider behind than in front in ratio 36 : 29. Clypeus 5/5.5 of the diameter of AME. Four bristles on edge of clypeus, one between AME. A row of bristles in middorsal line extending to thoracic groove where it ends in a pair. Irregularly arranged bristles to outside of eyegroup on each side, and to the inside of and behind PME and PLE. Rest of carapace clothed with short dark hair. Median band with light hair. *Cheliceræ* rather long. Basal joint 1.702 mm. long, with boss almost of the same color as the rest. Promargin with three teeth of which the distal tooth is quite small and close to the median tooth which is largest, being fully as large as the teeth of the retromargin. Retromargin with three equal, equidistant, strong teeth. Scopula on promargin only. A brush of hair on undersurface of cheliceræ. *Lip* wider than long in ratio 16 : 13, deeply excavated on sides in proximal half, articulated in middle to maxillæ and reaching to the middle of the latter. Anteriorly the lip is truncated to the width of the base. *Maxillæ* parallel to each other, wider in front than behind. Truncatures diverging, forming a right angle between them, with a brown scopula which does not extend to inner margin. Serrula marginal, weak and short, not reaching outer angle. *Sternum* oval, wider in front than behind, where it is pointed, longer than wide in ratio 48:40, truncated anteriorly to twice the width of the lip. First coxæ almost as far apart as second and fully as far apart as third coxæ. Fourth coxæ contiguous. All trochanters deeply notched. *Legs* 4123.

	Femur	Pat. + Tibia	Metatarsus	Tarsus	Total
I	3.198	3.690	2.255	1.435	10.578
II	2.788	3.362	2.255	1.435	9.840
III	2.624	2.952	2.460	1.353	9.389
IV	3.485	4.182	4.018	1.968	13.653
Palp	1.640	1.640		1.148	4.428

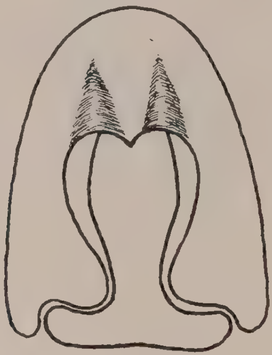

FIGURE 22, **Lycosa sancti thomasi** sp. nov. Epigynum.

Spines. First leg. Femur—dorsal 1-1-1, apical spine quite short and slender, prolateral 0-2, retrolateral 1-1-1 short, slender spines, ventral 0. Patella—dorsal 0-1 bristle, prolateral 1 fine bristle, retrolateral 1 fine bristle, ventral 0. Tibia—dorsal 1-1 fine bristles, prolateral 1 beyond middle, retrolateral 0, ventral 2-2-2. Metatarsus—dorsal 0, prolateral 0-1 near end, retrolateral 0-1 near end, ventral 2-2-1, apical spine small. *Second leg* same as first, except: Femur—prolateral 1-1, Patella—prolateral 1 spine in place of bristle. Tibia—prolateral 1-1. Metatarsus—prolateral 1-2. *Third leg.* Femur—dorsal 1-1-1, prolateral 1-1, retrolateral 1-1-1, ventral 0. Patella—dorsal 1-1 bristles, prolateral 1, retrolateral 1, ventral 0. Tibia—dorsal 1-1, prolateral 1-1, retrolateral 1-1, ventral 2-2-2. Metatarsus—dorsal 0, prolateral 1-1-2, retrolateral 1-1-2, ventral 2-2-1, all apical spines small. *Fourth leg* same as third, except: Femur—retrolateral 1-0-1. Metatarsus—ventral 1-2-2-1. *Palp.* Femur—dorsal 1-1-2, prolateral 0-1 at end, retrolateral 0-1 at end, ventral 0. Patella—dorsal 1-1, prolateral 1, elsewhere 0. Tibia—dorsal 1 in middle, prolateral 1, elsewhere 0. Terminal joint-dorsal 0, prolateral 2-1, retrolateral 1, ventral 0. First and second tarsi and metatarsi lightly scopulate to base. Third and fourth pair of legs without scopulæ, hair on ventral surface of third and fourth tarsi distinctly spinose. Trichobothria in two rows on tarsi and tibæ, in one row on metatarsi. Claws 3. Upper claws with 5 teeth, third claw smooth. Epigynum typical, as shown in Figure 22. *Color in alcohol:* Eyegroup set in black. Carapace brown with a yellow median band beginning halfway between PME and PLE, widening behind the latter, slightly constricted by the cephalothoracic sulci, thence gradually narrowing to posterior margin. Sides of band not sharply defined. A narrow marginal black line and a little above it an indistinct yellow narrow band with irregular outlines. Cheliceræ rufous. Legs light brown above, with femora with two transverse dark bands of irregular outline. Below, lighter, almost yellow. Sternum with median longitudinal dark band. Tibiæ somewhat darker at both ends. Abdomen above greyish-brown with indistinct darker ∧-marks. Below, uniformly light grey-brown. One female and one immature specimen from St. Thomas in the collection of Dr. Shoemaker.

FAMILY ATTIDÆ

Corythalia iridescens sp. nov. (Figures 23 and 24).

A male. Total length 6.240 mm. Carapace 3.0625 mm. long, 2.3625 mm. wide, 1.7500 mm. high. Head flat. Thoracic groove longitudinal, so situated that an imaginary line tangent to PLE cuts it in middle. Posterior declivity steep, slightly longer than level portion of thorax, the ratio of the distance between the end of the head and the beginning of the declivity to the length of the latter being 13:15. Eyegroup 1.6625 mm. long, first row of eyes 2.1875 mm. wide, second row 1.9250 mm., third row 2.1875 mm. PME nearer PLE than ALE in ratio 8:12. Ratio of eyes AME : ALE : PME : PLE = 17 : 11 : 2.25 : 10. Clypeus 8/17 of the diameter of AME. Viewed from in front, the first row of eyes is very gently curved upward by upper margins. AME slightly but distinctly separated from each other, and twice as much from ALE, *i.e.* 2.5/11 of the diameter of the latter. Clypeus irregularly fringed with brown hair. Four long bristles on edge of clypeus, three bristles in a triangle between and somewhat in front of AME, and two bristles on each side in front of and slightly to the side of AME. Several bristles behind the first row of eyes, others on each side of the eyegroup and scattered on head and on the level portion of the thorax. Eyes in black spots, but rest of chitin of carapace brown. Iridescent white scales behind first row of eyes, between ALE and PLE, behind PLE of each side in a triangular spot on the level portion of thorax and on the broad marginal band extending also to posterior edge. A median, rhombic spot between the two white triangular spots is formed by brown hair. Brown scales on sides of carapace. *Cheliceræ* stout, normal, with normal short fang. Promargin with two teeth fused at base, retromargin with a single, very broad, blunt tooth almost of the shape of a lobe, but not of the bicuspid type characteristic for the group of Fissidentati. Moreover, the female has a typically sharp, pointed tooth. A very scant scopula on promargin only. Dorsal surface of cheliceræ (outside) clothed with long stiff hair. *Lip* twice as long as wide, reaching beyond middle of maxillæ, articulated to maxillæ in middle where it presents a peculiar transverse, straight ridge. Anteriorly to the ridge the lip is triangular, narrowly truncated at end. *Maxillæ* parallel to each other, considerably widened in front, outer angle rounded. Scopula thick, brown, extending to the level of the lip. Serrula almost marginal, rather short, not reaching outer angle. *Sternum* oval, slightly convex, wider behind than in front where it is narrowed down to less than the width of the lip. Nevertheless the first coxæ are much farther apart than the width of the lip. Fourth coxæ contiguous. *Legs* 3124.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	1.998	1.332	1.517	1.332	0.666	6.645
II	2.072	1.258	1.332	1.221	0.629	6.512
III	2.442	1.332	1.517	1.665	0.740	7.696
IV	1.961	0.851	1.258	1.702	0.666	6.438
Palp	1.258	0.703	0.407		0.888	3.276

First and second pairs of legs heavier than the others, and first, second and third pairs with heavy black fringes. On the femur the fringe begins in the middorsal line and extends over the entire prolateral surface almost to midventral line. On the first leg there is in addition to this fringe only a small fringe on the ventral surface of the patella. On the second and third legs there is a heavy midventral and a light middorsal fringe on both patella and tibia. The third metatarsus has also a middorsal and a midventral fringe. Generally speaking, the fringes are heaviest on the third leg. The fourth leg has no fringes. Scattered white iridescent scales are present on all joints of all legs. Trichobothria in two rows on tibiæ and tarsi, in one row on metatarsi. Ungual tufts heavy. Claws 2, dissimilar. Proclaw with a long series of teeth, retroclaw with 5 teeth only.

Spines. First leg. Femur—dorsal 1-1-2, prolateral 0-0-2, elsewhere 0. Patella—dorsal 1-1 long bristles, prolateral 1, retrolateral 1, ventral 0. Tibia—dorsal 1-1 long bristles, prolateral 1-2, retrolateral 1-1, ventral 2-1r-2, first pair near base. Metatarsus—dorsal 0, prolateral 1-1, retrolateral 1-1, ventral 2-2. *Second leg* same as first, except: Femur—retrolateral 0-0-1, Tibia—prolateral 1-1-1, ventral 1r-2-2. Metatarsus—dorsal 1-0 near base. *Third leg.* Femur—dorsal 1-1-1, prolateral 0-0-2, retrolateral 0-0-1, ventral 0. Patella—dorsal 1-1 bristles, prolateral 1, retrolateral 1, ventral 0. Tibia—dorsal 1-1, the first a spine, the second a bristle, prolateral 0-1-1, retrolateral 1-1-1, ventral 1p-2. Metatarsus—dorsal 1-0 near base, prolateral 1-2, retrolateral 1-2, ventral 2-2. *Fourth leg* same as third, except: Femur—prolateral 0-0-1. Tibia—prolateral 1-1-1. Metatarsus—prolateral 1-1-2, retrolateral 1-1-2. *Palp.* Femur with a dorsal, a prolateral and a retrolateral black brush, the two latter ones appearing as a single ventral brush when the palp is viewed from the side. There is a middorsal spine present at the end of the femur. Patella entirely white above, the color due to long white hair and white iridescent scales. A middorsal bristle at end. Tibia with a retrolateral and a ventral apophysis (Figure 23 RA and VA), and with long, brown prolateral bristles. Copulatory apparatus with curved terminal embolus. *Abdomen* clothed above with brown recumbent hair, white iridescent scales and scattered long brown bristles. Below with a few hairs. Spinnerets cylindrical of equal length. Anterior pair heavier and darker. Colulus wanting. *Color in alcohol:* Above, generally yellowish-brown with hair and scales as described. Below, light yellow. Venter of abdomen dusky with two yellow longitudinal bands and a row of small round yellow spots on each side in the dark field.

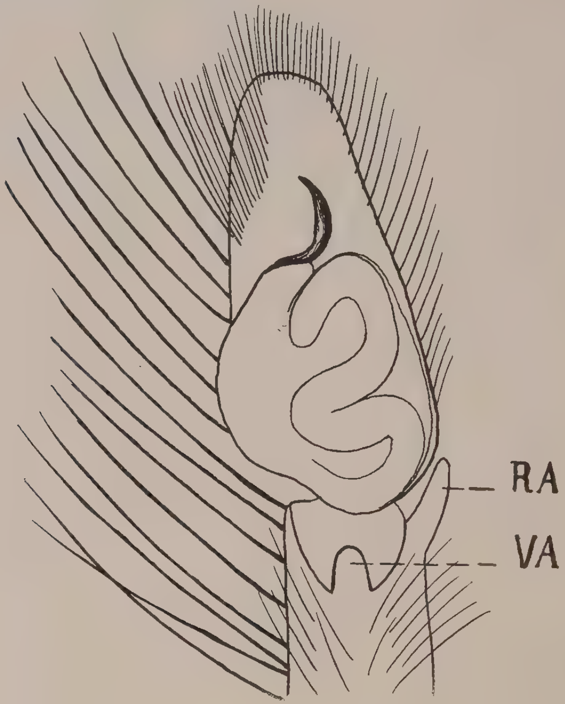

FIGURE 23, **Corythalia iridescens** sp. nov. Left palp of male from below.

This is the largest of the males. Other specimens are much smaller, measuring about 4.6 mm. in length and slightly darker, with venter of abdomen uniformly dark grey, but with the small yellow spots as in the larger specimen. Other characters same as in the type.

6) Female. Total length including spinnerets 7.2 mm. Carapace 3.157 mm. long, 2.460 mm. wide, 1.406 mm. high. Eyegroup 1.600 mm. long. First row of eyes 2.173 mm. wide, second row 2.050 mm., third row 2.255 mm. PME nearer PLE than ALE in ratio 8 : 11. Ratio of eyes AME : ALE : PME : PLE = 17 : 11 : 2.5 : 10. Cheliceræ as in male, but the tooth on the retromargin sharp, conical. *Legs* 3412.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	1.665	1.147	1.147	1.073	0.555	5.587
II	1.665	0.999	0.999	0.999	0.481	5.343
III	2.109	1.110	1.295	1.406	0.592	6.512
IV	1.887	0.851	1.184	1.443	0.592	5.957
Palp	0.999	0.518	0.444	0.814		2.775

Spines. First leg. Femur—dorsal 1-1-1, prolateral 0-0-2, retrolateral 0-0-1, ventral 0. Patella—dorsal 1-1 bristles, prolateral 1, retrolateral 1, ventral 0. Tibia—dorsal 1-1 bristles, prolateral 1-2, retrolateral 1-1, ventral 2-1r-2, first pair near base. Metatarsus—dorsal 0, prolateral 1-1, retrolateral 1-1, ventral 2-2. *Second leg* same as first, except: Femur—retrolateral 0-0-2. Tibia—prolateral 1-1-1, ventral 1r-2-2. *Third leg.* Femur—dorsal 1-1-2, prolateral 0-0-2, retrolateral 0-0-1, ventral 0. Patella—dorsal 1-1 bristles, prolateral 1, retrolateral 1, ventral 0. Tibia—dorsal 1-0 near base, prolateral 1-1, retrolateral 1-1-1, ventral 1p-2. Metatarsus—dorsal 1-0 near base, prolateral 1-2, retrolateral 1-2, ventral 2-2. *Fourth leg* same as third, except: Femur—dorsal 1-1-1, prolateral 0-1. Metatarsus—prolateral 1-1-2, retrolateral 1-1-2. Black fringes on legs wanting, but in the palp the dorsal surface of the patella is thickly clothed with white hair and white iridescent scales as in male. Femur of palp with a middorsal spine at end, patella with a middorsal bristle at end, tibia with prolateral bristles. There are bristles also on the terminal joint. Epigynum shown in Figure 24, with two spherical receptacles of dark color. Palpal claw wanting. Coloration and distribution of scales as in male, but dark spot on carapace of deeper hue and more distinct. Very

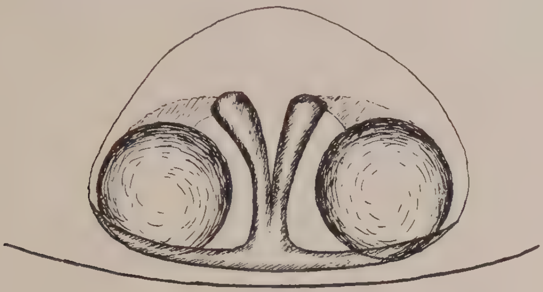

FIGURE 24, **Corythalia iridescens** sp. nov. Epigynum.

distinct are also a pair of brown spots on the back of the abdomen a little behind middle. Numerous females and males collected by Mr. Shoemaker in St. Thomas.

Stridulattus gen. nov.

Carapace low, with longitudinal thoracic groove. Eyegroup almost parallel, slightly wider in front than behind, wider than long. PME behind middle. Clypeus less than radius of AME. Cheliceræ normal, but with stridulating ridges on retrolateral surface, corresponding to a peculiar tubercle at base of femur of palp, bearing cuspules with stiff setæ. Promargin with two teeth, retromargin with a single, cone-shaped tooth. Lip twice as long as wide. Maxillæ dilated at end. Sternum truncated in front and first coxæ wide apart. First pair of legs slightly heavier than the others. Fourth patella plus tibia longer than third patella plus tibia. Legs 4132. First tibia with 1 prolateral and 1p-1p-2 ventral spines. First metatarsus with 2-2 ventral spines. Second tibia only with a pair of ventral apical spines. Fourth tibia only with six apical spines as is the case also with the fourth metatarsus. Belongs in the vicinity of *Heliophanus*. Type **S. stridulans.**

Stridulattus stridulans sp. nov. (Figures 25-28).

Male. Total length including spinnerets 5.75 mm. Carapace 2.7125 mm. long, 2.0125 mm. wide in middle, 1.1350 mm. high in region of PLE. Since this is the highest region, the carapace must be regarded as being rather low. Thoracic groove longitudinal, its anterior end slightly behind imaginary line tangent to PLE. Head almost flat. Between PLE a slight depression. Posterior declivity begins halfway behind eyegroup and is rather gentle. Eyegroup 1.332 mm. long, thoracic portion 1.517 mm. long. First row of eyes 1.591 mm. wide, second row 1.406 mm., third row 1.517 mm. 0:3 nearer PLE than ALE in ratio 8:12 if measured to edge of cornea, or in ratio 8:8 if measured to base of ocular cylinder. Ratio of eyes AME : ALE : PME : PLE = 13 : 7 : 2 : 7. AME narrowly but distinctly separated from each other by 1/13 of their diameter and from ALE by 2/13 of their diameter. Viewed from in front, first row of eyes is straight by upper margins. Clypeus 4/13 of the diameter of AME, heavily clothed with white hair obstructing the view of the base of cheliceræ. No scales either on body or legs. Pedestals of anterior eyes surrounded by short, rodlike, greyish-brown hair. Brown bristles on sides of head and behind anterior row without definite arrangement. A marginal band on carapace clothed with white hair extending from base of cheliceræ to posterior edge. Clypeal band of white hair extends on each side of head as far as the end of the eyegroup, leaving brown color between the latter and the marginal band. Head with white hair extending in a median, not clearly defined band to posterior edge of carapace. Chitin of head much darker than chitin of thoracic portion, so that in alcohol carapace appears brown with black head. *Chelicerœ* short and stout, anteriorly sparsely clothed with dark hair, with stridulating ridges on retrolateral surface as shown in Figure 25. Promargin with two teeth, retromargin with a single cone-shaped, well developed tooth. A scant scopula of long hair on promargin, and a single row of hairs on retromargin. Fang normal. *Lip* very long, reaching considerably beyond the middle of maxillæ to which the lip is articulated in its middle. Basal half of lip with more or less parallel sides. From here on the lip narrows down toward the end where it is truncated. The lip is longer than wide in middle in ratio 18:9. *Maxillœ* wider at end, with short, diverging truncatures. Outer angle rounded, with peculiar short hook directed forward (Figure 25). Outer edge slightly concave. Scopula of the normal type, brown. Serrula considerably beyond anterior margin, visible only from above, not parallel to edge, but obliquely

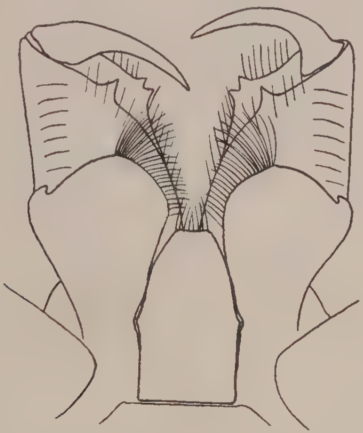

FIGURE 25, **Stridulattus stridulans** gen. et sp. nov. Cheliceræ, lip and maxillæ of male. Note the stridulating ridges.

diverging from outer end of scopula to base of outer hook. *Sternum* truncated in front to slightly more than width of lip, pointed behind, in front of fourth coxæ which are contiguous. Sternum longer than wide in ratio 32:24. *Legs* 4132. First leg distinctly heavier than the others.

	Femur	Patella	Tibia	Metatarsus	Tarsus	Total
I	1.665	1.036	1.221	0.962	0.481	5.365
II	1.406	0.962	0.962	0.888	0.444	4.662
III	1.406	0.740	0.962	0.962	0.629	4.699
IV	1.776	0.888	1.369	1.441	0.629	6.103
Palp	0.851	0.296	0.259	0.851		2.257

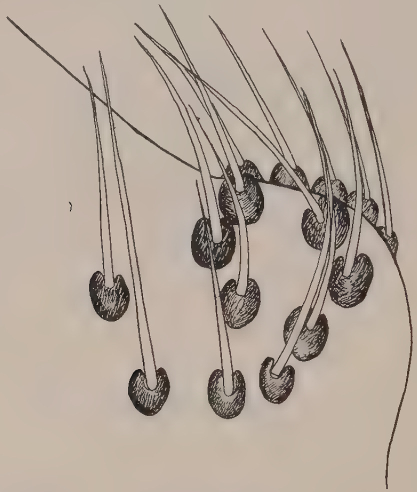

FIGURE 26, **Stridulattus stridulans** gen. et sp. nov. A portion of the tubercle of the femur of the palp of the male with stridulating setæ.

Spines. First leg. Femur—dorsal 1-1-1 long spines, prolateral 0-2, elsewhere 0. Patella—dorsal 1 bristle at end, elsewhere 0. Tibia—dorsal 1-1 bristles, prolateral 0-1 about 1/5 from distal end, retrolateral 0, ventral 1p-1p-2, first spine near base, second in middle. Metatarsus—dorsal 1-1 bristles, ventral 2-2, elsewhere 0. *Second leg* same as first, except: Femur—prolateral 0-1. Tibia—ventral 0-0-2. *Third leg.* Femur—dorsal 1-1-1 long spines, prolateral 0-1-1, retrolateral 0-0-1, ventral 0. Patella—dorsal 1 bristle at end, elsewhere 0. Tibia—dorsal 0, prolateral 1, retrolateral 1, ventral 0-2 apical spines. Metatarsus—dorsal 0, prolateral 0-2 at end, retrolateral 0-2 at end, ventral 0-2 at end. *Fourth leg* same as third, except: Femur—prolateral 0. Tibia—prolateral 0, retrolateral 0. *Palp* without spines, but with 1-1 dorsal bristles on tibia. Femur of first leg with strongly convex dorsal surface.

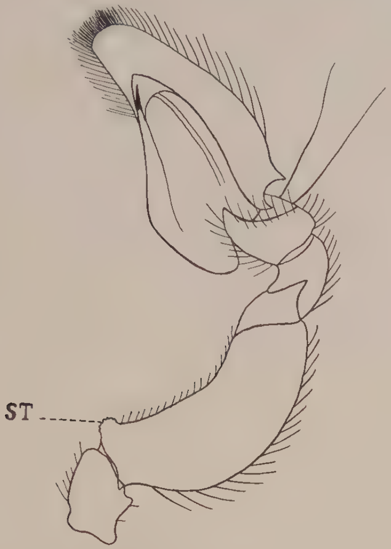

FIGURE 27, **Stridulattus stridulans** gen. et sp. nov. Left palp of male viewed from the retrolateral side. Note stridulating tubercle at ST.

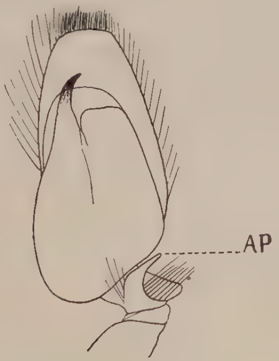

FIGURE 28, **Stridulattus stridulans** gen. et sp. nov. Left palp of male from below. Note tibial apophysis at AP.

Trichobothria mostly lost, apparently in a single row on all tibiæ, metatarsi and tarsi. Ungual tufts well developed. Claws two, dissimilar. Proclaw with a series of 11 teeth, retroclaw with from

2 to 4 teeth. Legs clothed with simple hair. Femur of palp curved dorso-ventrally, furnished at base with a tubercle (Figure 27 ST) with peculiar setigerous cuspules for stridulation against the ridges of the cheliceræ. A few of these setæ are shown in Figure 26 highly magnified. Tibia of palp with a retrolateral apophysis which in ventral view appears as a spine. Cymbium with dorsal cone-shaped protuberance at base. Copulatory apparatus shown in Figures 27 and 28. *Abdomen* clothed with white and rust-red hair. The latter forms a median dorsal longitudinal band and a lateral longitudinal band on each side of abdomen. Ventral surface of abdomen clothed with very short hair. Spinnerets about equal in length and diameter, cylindrical. *Color in alcohol:* Head almost black, carapace brown, legs lighter brown. Abdomen with median and lateral longitudinal rust-red bands. Below, greyish-yellow. One male taken by Mr. Shoemaker in St. Thomas in July 1915.

University of Porto Rico,
Rio Piedras, Porto Rico,
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3001

The prevention and cure of tetany by oral administration of magnesium lactate.

W. F. WENNER. (Introduced by W. W. Swingle).

[*From the Osborn Zoological Laboratory, Yale University, New Haven, Conn.*]

It was demonstrated in a recent series of experiments[1] that strontium lactate administered orally to parathyroidectomized dogs greatly delays the onset of tetany, and may even bring about a permanent cure if the treatment is long continued. Preliminary experiments, carried out in conjunction with the strontium work, indicated that magnesium salts act similarly to strontium. The present paper is concerned with this phase of the work.

If parathyroidectomized dogs are given a 5 per cent solution of magnesium lactate in 100 cc. doses three times daily by stomach tube, they may be kept free from tetany for long periods, occasionally for three weeks. Some of the animals may develop tetany after eight or ten days. Once tetany becomes manifest, and the serum calcium falls below 7 mg. per 100 cc., the magnesium exerts little effect except to allay the more violent symptoms, probably through its anesthetic effect. However, if the animal is fed a pint or more of milk each day, along with the magnesium, the tetany soon disappears, generally within three or four days, and if the milk is discontinued the dog remains normal and shows no tetany symptoms so long as the magnesium is administered. Examination of the serum calcium shows that the calcium has risen to 7.5 or 8 mg. per 100 cc., which is above the level at which tetany becomes manifest in dogs.

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[1] Swingle, W. W., and Wenner, W. F., *Am. J. Physiol.*, 1926, lxxv, 372-8.

The experiments indicate clearly that magnesium, *per se,* exerts little influence on tetany except through its action in sparing calcium. Somehow, the magnesium prevents the serum calcium from falling below the tetany level. A striking illustration of the sparing action of magnesium on calcium is shown in the following experiment. Parathyroidectomized dogs were allowed to develop tetany and then given the regular magnesium treatment. The animals died within a few days although the more violent symptoms of tetany were held in check. On the other hand, if tetany dogs are given several hundred cc. of calcium lactate by mouth, and this is followed by magnesium treatment, many of the animals do not again show tetany symptoms. The calcium raises the level of this salt in the blood and the magnesium somehow serves to maintain the level above 7 mg. per 100 cc. In the absence of magnesium, the calcium level of the blood soon falls and tetany appears.

It is a curious fact that if magnesium treated dogs are kept free from tetany for forty days, a large per cent of them permanently recover from tetany and may be placed on a heavy meat diet without ill effects. Thus ten dogs were used in this experiment; animals 2, 3, 7, 8 and 9 completely recovered and readjusted themselves to loss of their parathyroids. Dog 6 remained free from tetany for ten days after operation but escaped from the laboratory before completion of the experiment. All of the animals developed tetany some time during the experiment, for if the tetany symptoms did not develop spontaneously by the third week, the magnesium was discontinued and the animals were fed meat until symptoms appeared.

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DEPRESSION OF OXIDATIVE METABOLISM AND RECOVERY FROM DILUTE POTASSIUM CYANIDE

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TWO FIGURES

INTRODUCTION

Although the cyanides have been widely used as general depressants of protoplasmic activity, particularly of oxidative metabolism, much of the nature of the mechanism of their effects remains obscure. Certain similarities between the effects of cyanides and deficient oxygen supply were early recognized, and Geppert(1), after extensive work with mammals, concluded: ''Die Blausäure hemmt die Verbrennungen im Thierkörper.'' Considerable evidence has accumulated which supports Geppert's conclusion. For an excellent review of the literature on the effects of cyanides on oxygen metabolism, see Hyman(2).

Depression of oxidations in protoplasm by cyanides in appropriate concentrations is apparently readily reversible. However, the phenomena attendant upon recovery do not appear to have been studied rigorously. Thus Vernon(3) found recovery of normal respiratory rate in the perfused kidney of the dog after treatment with HCN; Shroeder(4) noted recovery of normal rate of oxygen consumption in Aspergillus niger after exposure to certain concentrations of KCN; Onaka(5) measured the oxygen consumption of red blood corpuscles of geese and found recovery complete one and one-half hours after depression by KCN; Hyman(6, 7, 2) found recovery in sponges, coelenterates, flatworms, and annelids after depression with KCN; Hyman's recovery determinations were made a number of hours after removal from KCN and do not represent a continuous record of the recovery phenomena; Child(8) showed that recovery of normal rate of carbon-dioxide production in flatworms is attained gradually after exposure to certain concentrations of KCN; with weaker solutions, M/20,000, Child records data indicating that the rate of carbon dioxide production may rise above the normal when the KCN is removed; Allen(9) states that recovery of rate of oxygen consumption in flatworms after depression by KCN is rapid and gives data indicating that the recovery rate may exceed the normal.

The data on depression of oxygen consumption and recovery from KCN presented in this paper are in part assembled from experiments in an investigation of the comparative effects of cyanide and certain other agents which will appear in a later paper. While they are confined chiefly to the effects of a single concentration of KCN, namely, M/25,000, they include a comparatively large number of experiments and give a fairly complete record of the effects of KCN acting for a twenty-four-hour period and the changes in rate of oxygen consumption during recovery. The findings of Hyman(2), Child(8), and Allen(9) are confirmed and extended, and new data on recovery are recorded which appear to have an important bearing on the problem of the effect of cyanide on the organism and its similarity to the effect of low oxygen tension. The number of experiments is sufficient to justify a mathematical treatment of the correlation between the normal rate of oxygen consumption and the degree of depression in cyanide, and thus new evidence of the basis of differential susceptibility appears.

MATERIAL AND METHODS

The material used in this study was Planaria dorotocephala, a fresh-water flatworm abundant in springs in certain parts of the country.[1] This form has certain advantages for such an investigation, among which is that it is easily kept in normal condition and its physiological state controlled in the laboratory. In this laboratory the animals are kept in large agate-ware dishpans in tap-water, and fed twice or three times a week with fresh beef liver. The state of nutrition is thus easily controlled, and animals of the same size selected from the same stock pan show a high degree of uniformity as regards physiological condition soon after being brought into the laboratory.

The method consisted in measuring the oxygen consumed per unit of weight per unit of time, of a lot or lots of forty or more animals of approximately the same size and taken from the same stock pan, in well-aerated tap-water, then replacing the water with freshly made KCN solution and measuring the oxygen consumption of the animals in the KCN solution during the first two hours of exposure. The cyanide solution was then replaced by fresh, and the oxygen consumption taken again at certain periods as the exposure continued, the process of replacing the KCN solution with fresh being repeated at least every four hours except at night, when the animals remained in the same solution eight or ten hours. All air was excluded from the flasks throughout the exposure to KCN except during the process of changing the solutions and obtaining samples for oxygen analysis. The animals were then washed in four or five changes of water for eighteen to thirty minutes and the oxygen consumption taken in tap-water during the first hour following or at certain other periods during recovery. In all cases the animals were weighed at the close of the experiment, the method of weighing being described elsewhere in detail(10). With few exceptions, the concentration of KCN employed was M/25,000, made up in eight liter quantities from a freshly made M/1000 solution. All solutions and water used were well aerated and contained adequate oxygen supply.

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[1] An abundant supply of material was received from the Hull Zoölogical Laboratory, University of Chicago, through the kindness of Dr. Libbie Hyman. The methods of feeding and keeping the stock in normal condition were essentially those worked out in the Chicago laboratory, accounts of which may be found in many papers of Child, Hyman, and others published from that laboratory.

For determining the oxygen content of the water and cyanide solutions the Winkler method was used. This method is well known and needs no extended comment here. As employed in this work a brief statement of its reliability as regards the oxygen content of given samples of water seems necessary. Duplicate samples of water were drawn simultaneously from a container of well-stirred tap-water and the oxygen content of each taken. The results of eight such tests in terms of cubic centimeters of oxygen per liter were:

(1) 5.96—5.96
(2) 5.21—5.24
(3) 6.13—6.14
(4) 5.58—5.59
(5) 4.63—4.67
(6) 6.42—6.40
(7) 5.91—5.93
(8) 5.97—6.00

The extreme variation in these eight tests is therefore 0.04 cc. which is within the limit of accuracy of the method.

Measurements of the oxygen content of the cyanide solutions were carried out in the presence of KCN. Since the Winkler method is an iodometric method, it is to be expected that the KCN and iodine react with consequent error in the determinations of dissolved oxygen. In M/25,000 KCN this error is considerable. To a quantity of tap-water was added sufficient KI solution of iodine to color. The solution was then divided and to one part was added KCN to make a M/25,000 solution. Equal volumes of each were then repeatedly titrated against M/100 sodium thiosulphate. The end point of the solution containing KCN was not sharp, but sufficiently marked to show that the titrable iodine present was distinctly less than in the water containing iodine. The calculations of the oxygen content of the cyanide solutions then represent the oxygen present minus the oxygen equivalent of the iodine absorbed by the KCN. Since this error occurs in both the samples drawn from the original solutions and in those drawn from the flasks containing the animals, no violence is done to the determinations of the oxygen consumption of the animals in cyanide. It is evident, however, that the Winkler method cannot be used to compare the oxygen content of cyanide solutions with that of water.

Known possible sources of error arising from manipulation of the animals were controlled as follows: Temperature: All tests in a given experiment were conducted at the same temperature maintained within less than one degree. Time: The actual time over which a test was taken was accurate within less than two minutes. Motor activity: This was reduced to a minimum by keeping the animals in darkness during the tests, by having the temperature of the experiment slightly below that of the stock pan from which the animals were taken, by introducing the animals into the flasks some hours before the tests were to be made, and, in many cases, by beheading the animals. Under such conditions, motor activity is slight and insufficient to account for differences in oxygen consumption of the magnitude recorded. Hydrogen-ion concentration: Tap-water freshly drawn for these experiments has a hydrogen-ion concentration of pH 7.0 to pH 7.2 (phenol-red method). After standing for twelve hours, the alkalinity increases to pH 7.4 to pH 7.6. No differences in oxygen consumption of animals in freshly drawn tap-water when compared with that of animals in water that had stood for twelve hours or longer was noted. The addition of M/25,000 KCN increases the alkalinity slightly, pH 0.4. A similar change of pH in tap-water being without appreciable effect, it was considered unnecessary to bring all solutions and the water used in an experiment to the same pH value.

The details of manipulation of the animals, containers, etc., have been described at some length in an earlier paper (11). An examination of the data given in the tables in the present paper (column 1, the oxygen consumption of animals in water) shows that the variation in normal rate of oxygen consumption between comparable groups of animals is small.

In the thirty-three such groups given here plus twenty-six groups among unpublished data, the extreme variation within any group is but 0.00007 cc. of oxygen per milligram per two hours, which occurs in only two cases. The greatest frequency of variation is 0.00003 cc. Differences of 0.00003 cc. or less occur in forty-one of the fifty-nine groups. It will be apparent later that the standardization of both material and methods is sufficiently accurate for the purpose of this study.

DATA

Figure 1 is a graph showing the average results of experiments on the effect of M/25,000 KCN on the oxygen consumption of P. dorotocephala during the first four hours of exposure and recovery during the first six hours following. The depression during the exposure to cyanide is given in terms of the average per cent of the normal and is based on the data from eighty-six experiments. The recovery during the first hour after removal from the KCN is based on sixty-three experiments, the recovery for one hour, one-half hour after removal on twelve experiments, and the oxygen consumption for a two-hour period six hours after removal from the cyanide on six experiments. A graph of the effect of M/50,000 KCN during a similar period is superimposed. All experiments carried out are included.

Figure 2 is a graph showing the oxygen consumption at stated periods during twenty-four hours exposure to M/25,000 KCN and the first hour of recovery and is based on eleven experiments.

The tables give the complete data from which figures 1 and 2 were drawn and include all the data that the writer has obtained.

Summarizing, ninety-two experiments show, 1) that depression of oxygen consumption produced by KCN in the concentrations used occurs chiefly during the first two hours of exposure; 2) the rate of oxygen consumption does not change greatly during the remainder of a twenty-four-hour exposure; occasionally a slight temporary acclimation during the second two-hour period is indicated; 3) on removal from KCN, the rate of oxygen consumption rises above normal during the first hour (six exceptions in ninety-two cases); 4) the extent of the rise above the normal is independent of the duration of exposure to the cyanide; 5) the return downward to normal rate requires at least six hours.

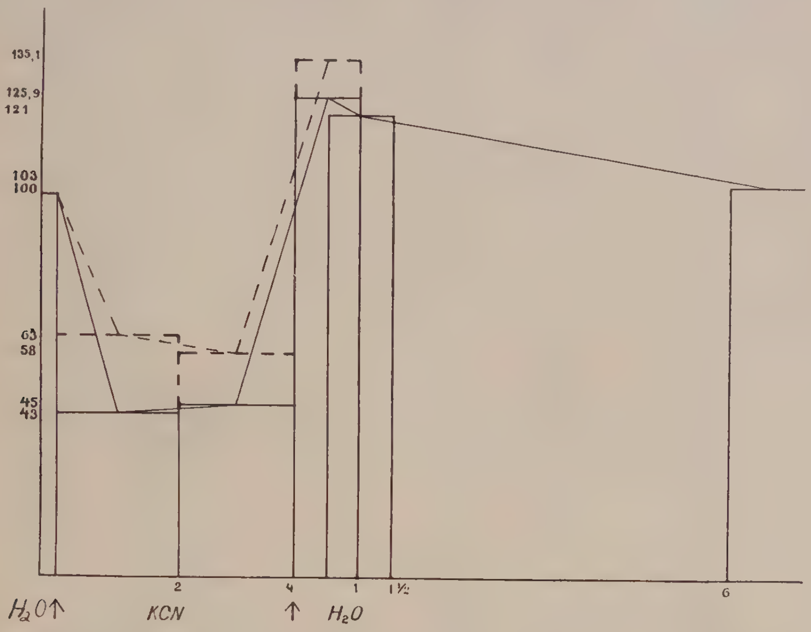

Fig. 1 Oxygen consumption, normal four hours in KCN, and recovery up to eighth hour. Normal is the average per two hours of a two-to-sixteen-hour test counted as 100 per cent and plotted as ordinate. Other ordinates are average per cent of normal. Abscissae are hours. Broken lines are results with M/50,000 KCN. Solid lines are results with M/25,000 KCN.

The supernormal rate of oxygen consumption noted in (3) above requires some inquiry as to the possibility of factors other than intrinsic oxidative metabolism being concerned. Dealing first with the possibility of increased motor activity after exposure to cyanide, it may be stated definitely that no differences in behavior between animals after exposure to KCN and control animals can be observed. The manipulation of washing the animals taken from the cyanide solution was simulated in trials with animals that had not been subjected to KCN. In each case they distributed themselves about on the interior of the flasks within a few minutes and remained practically motionless for long periods unless violently disturbed. The possibility of a stimulating effect of minute traces of KCN remaining in the animals after washing is next to be considered. There is no evidence elsewhere in these

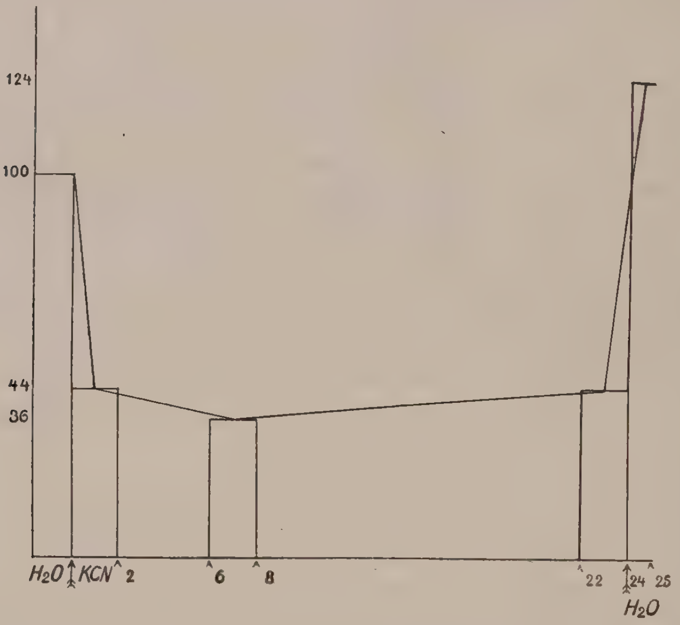

Fig. 2 Oxygen consumption, normal, twenty-four hours in M/25,000 KCN, and first-hour recovery. Ordinates and abscissae as in figure 1.

results of any primary acceleration of oxygen consumption in KCN solutions. Hyman(2, pp. 368, 369) gives some data showing a very slight acceleration of oxygen consumption in Planaria during the first half-hour of exposure to M/150,000 KCN. In the data presented here, however, the differences between the supernormal rate of the first hour of recovery and the original normal rate are in general much greater and the supernormal rate persists for at least six hours. The two series of data, i.e., that of Hyman and that

TABLE 1

Oxygen consumption per milligram per two hours. In 0.00001 cc. Normal over two to sixteen hours, four hours' exposure to KCN, and first hour recovery in water. Bracketed data are of experiments run parallel. Starred figures are exceptions referred to in the text

LENGTH	TIME SINCE FEEDING	WEIGHT	HEADS OR BEHEADED	TEMPERATURE	CONCENTRATION OF KCN	OXYGEN CONSUMPTION WATER	KCN		WATER
							First two hours	Second two hours	First hour
mm.	*days*	*mg.*							
20	6	340	H.	20	M/50,000	47	31	18	60
18	14	495	H.	20	“	47	39	40	64
18	2	636	H.	20	“	45	29	25	68
18	2	586	H.	20	“	44	23	24	67
18	2	619	H.	20	“	54	30	23	67
18	2	491	H.	20	“	42	26	31	62
18	2	586	H.	20	M/25,000	57	29	26	62
18	1	490	H.	20	“	53	27	18	82
18	2	494	H.	20	“	50	26	20	76
18	3	515	H.	20	“	46	34	38	56
18	2	522	H.	20	“	49	29	31	70
18	2	637	H.	20	“	47	24	21	47*
18	2	471	H.	20	“	53	22 (ave.)		63
25	2	336	H.	21	“	44	27	25	54
7	2	167	H.	21	“	56	23	31	60
7	2	169	H.	21	“	52	26	33	46*
25	2	515	H,	23	“	43	26	27	58
7	2	253	H.	23	“	53	31	29	85
7	2	231	H.	23	“	49	29	22	69
25	2	420	H.	24	“	45	26	24	52
7	2	220	H.	24	“	54	40	30	72
7	2	227	H.	24	“	52	42	29	70
20	1	458	H.	20	“	54	23	25	82
6	1	413	H.	20	“	63	25	31	101
20	1	470	Behead.	20	“	57	19	23	67
20	3	518	Behead.	20	“	53	19	18	70
6	3	344	Behead.	20	“	54	23	28	98
20	3	501	H.	20	“	57	14	22	91
18	3	455	Behead.	20	“	48	20	20	51
18	3	439	Behead.	20	“	49	18	16	69
18	3	510	Behead.	20	“	49	23	17	65
18	3	509	Behead.	20	“	50	19	19	51
18	3	493	Behead.	20	“	42	18	18	58
18	3	546	Behead.	20	“	53	19	18	57
18	3	485	H.	20	“	60	22	5	68
18	3	522	Behead.	20	“	32	19	31	54
18	3	510	H.	20	“	34	19	12	65
18	4	465	Behead.	20	“	50	23	24	41*
18	4	516	H.	20	“	55	28	24	40*
18	7	449	Behead.	20	“	44	18 (ave.)		45
18	7	458	H.	20	“	47	19 (ave.)		55
18	1	501	Behead.	20	“	49	17	14	50
18	1	527	H.	20	“	49	17	14	55
16	4	429	H.	20	“	57	22	10	83
16	4	551	H.	20	“	51	19	16	66
16	4	468	Behead.	20	“	42	21	16	61
16	4	485	Behead.	20	“	39	15	19	56
16	7	481	Behead.	20	“	40	18	22	60
16	7	429	Behead.	20	“	43	22	23	65
18	4	496	Behead.	20	“	48	17	21	63
18	4	529	Behead.	20	“	47	20	17	54
16	10	531	Behead.	20	“	45	17	17	38*
16	10	586	Behead.	20	“	42	16	16	37*
16	6	537	Behead.	20	“	36	20	21	43
16	6	445	Behead.	20	“	38	19	24	49
18	5	457	Behead.	20	“	42	19 (ave.)		51
18	5	468	Behead.	20	“	42	19 (ave.)		53

given here, do not appear to be the effect of the same condition, namely, the presence of extremely dilute KCN.

DISCUSSION

It is probable that the depression of oxidative metabolism occurs very rapidly after admission of this concentration of KCN (M/25,000), since the total oxygen consumed during the first two hours of exposure is usually not greater and quite frequently less than that of the next two hours. Hyman(2) and Allen(9) in flatworms and Galigher(12) in annelids record similar results.

TABLE 2

Oxygen consumption per milligram per two hours. In 0.00001 cc. Normal over two to sixteen hours, four hours' exposure to KCN, first hour recovery in water, and one hour, one-half hour later. Beheaded animals. KCN, M/25,000. Temperature, 20°. Bracketed data are of experiments run parallel.

LENGTH	TIME SINCE FEEDING	WEIGHT	OXYGEN CONSUMPTION WATER	KCN		WATER	
				First 2 hours	Second 2 hours	First hour	One-half hour later
mm.	*days*	*mg.*					
⎰ 16	4	538	40	14	21	57	
⎱ 16	4	552	41	15	21		55
⎰ 18	3	597	41	17	23	60	
⎱ 18	3	622	40	17	21		48
⎰ 18	1	646	47	23	20	56	
⎱ 18	1	647	47	25	20		49
⎰ 16	6	619	45	18	19	61	
⎱ 16	6	606	45	17	20		56
⎰ 16	3	622	46	18	23	54	
⎱ 16	3	597	47	18	23		62
⎰ 15	3	545	49	21	26	62	
⎱ 15	3	581	48	20	24		61
⎰ 16	3	604	45	17	16	51	
⎱ 16	3	638	41	16	13		50
⎰ 15	3	540	49	19	22	66	
⎱ 15	3	564	48	23	21		61
⎰ 15	2	517	49	21	35	70	
⎱ 15	2	507	51	21	30		57

That the minimum level of oxygen consumption so soon reached is maintained over a twenty-four-hour period without change except for slight fluctuations, may be considered as adequately demonstrated by the data. Suggestions as to the nature of the principal causes of these characteristics of the effect of KCN in such concentrations on oxidative metabolism must be sought in the type of curves shown in figures 1 and 2. Turning now to these curves, it is at once evident that they represent some sort of equilibrium, rapidly reached and maintained over a considerable period of time in the same concentration of cyanide. It is also evident that when this equilibrium is disturbed by the removal of the cyanide, oxidative metabolism of more than normal rapidity results almost immediately. These facts suggest that the reaction between this concentration of KCN and the organism whereby the oxidative metabolism is depressed is of the nature of an effect on some physical condition in the protoplasm rather than a chemical reaction. A number of arguments in support of this suggestion may be advanced. In the first place, Bodine (13) has shown that HCN penetrates living cells with considerable rapidity. Krehan(14), who has made an extensive study of the effects of KCN on the permeability of plant cells, showed that cyanide produces over a wide range of concentrations a gradually increasing and reversible increase in permeability. His curves are very dif-

TABLE 3

Oxygen consumption per milligram per two hours. In 0.00001 cc. Normal over two to sixteen hours, four hours' exposure to KCN, first hour recovery in water, one hour one-half hour later, and two hours six hours later. Beheaded animals. KCN, M/25,000. Temperature, 20°. Bracketed data are of experiments run parallel

LENGTH	TIME SINCE FEEDING	WEIGHT	OXYGEN CONSUMPTION WATER	KCN		WATER		
				First 2 hours	Second 2 hours	First hour	One-half hour later	Six hours later
mm.	*days*	*mg.*						
{ 15	6	461	49	23	24	60		53
{ 15	6	487	46	23	22		55	50
{ 14	5	453	45	25	22	63		51
{ 14	5	407	44	25	24		53	45
{ 18	2	492	46	20	23	56		43
{ 18	2	499	44	21	21		52	44

ferent from those in figures 1 and 2. It is therefore regarded as unlikely that the depression of oxidations by KCN is primarily due to its effect on the permeability of the cell. Secondly, if the effect producing the depression of oxidative metabolism were of the nature of a destructive chemical reaction between the KCN and oxidative enzymes or other catalysts, or the substrate, the reaction would be expected to continue so long as such substances are present, since the concentration of the cyanide is maintained unchanged throughout the twenty-four-hour period. In other words, if KCN depresses oxidative metabolism by forming a chemical compound with some substance or substances in the cell, oxygen consumption would be expected to diminish the longer the exposure, and recovery would be expected to occur only as rapidly as the destroyed substances are replaced. But a diminishing rate of oxygen consumption and a correspondingly slow recovery are not characteristic of a twenty-four-hour exposure to cyanide, and the data of Allen (9) indicate that the same is true for thirty-six-hour periods.

TABLE 4

Oxygen consumption per milligram per two hours over twenty-four hours. In 0.00001 cc. Normal over two to sixteen hours, first two hours' exposure to KCN, sixth to eighth hour in KCN, twenty-second to twenty-fourth hour in KCN, and first hour recovery in water. Beheaded animals. KCN, M/25,000. Temperature, 20°. Bracketed data are of experiments run parallel

LENGTH	TIME SINCE FEEDING	WEIGHT	OXYGEN CONSUMPTION WATER	KCN			WATER
				First 2 hours	Sixth to eighth hour	Twenty-second to twenty-fourth hour	First hour
mm.	*days*	*mg.*					
16	3	793	46	31		18	62
16	3	715	39	21		20	55
16	3	554	43	22		21	62
25	2	652	50	19	22	19	54
25	2	680	49	19	19	21	58
25	2	643	49	20	18	20	62
25	2	660	50	16	11	19	60
26	2	882	44	20	20	21	54
26	2	793	44	19	18	22	52
26	2	827	43	18	16	22	53
26	2	806	44	17	11	21	50

On the other hand, at least two types of physical effect of KCN may be mentioned, either of which appear to accord partially with the data. If this concentration of KCN produces a physical state or condition characteristic of the concentration of KCN and the particular protoplasm subjected to it, one should expect this condition to continue without material change so long as the concentration of KCN remains unchanged, until functional and structural breakdown result. If we assume that oxidations in protoplasm are related more or less intimately to the physical state of the protoplasm, and that cyanide affects this physical state in some way, its effect on oxidations becomes at least partially explicable. Neither of these assumptions is devoid of supporting evidence. That protoplasmic structure and state are of considerable importance in oxidative metabolism may be regarded as an accepted fact (see Lillie's discussion, 16, p. 52). It is also known that cyanides, like iodides and bromides, decrease the viscosity of certain colloids. In any interpretation of the effect of cyanides on oxygen metabolism these facts must be taken into account. In this connection it may be pointed out that Heilbrunn(15) showed that cyanide solutions arrest the viscosity changes attendent upon cell division, and suggests that the anesthetic action of KCN may be attributed to this effect. The anesthetic action of KCN and its depression of oxidations are not to be regarded as identical, however, and Heilbrunn's data do not necessarily constitute valid evidence supporting the suggestion that the physical effects of KCN may be primarily concerned in its depression of oxygen consumption.

A second possibility is that of adsorption on such surfaces as are concerned in catalyzing oxidations rendering them less active during the presence of this concentration of cyanide, but not destroying or permanently impairing their activity, i.e., acting as a negative catalyzer, but not by formation of a stable compound with the protoplasm or any of its components. Similar suggestions have been advanced by others, but in the absence of any information as to the nature of the catalytic agents concerned in respiration in Planaria, further discussion of this possibilty is profitless. The suggestions that the effect of this concentration of KCN on oxidative metabolism is primarily due to physical action and not to a chemical interaction between the cyanide and components of the cell are admittedly indefinite in form. But their indefinite form is a point of merit, for the present status of the problem of biological oxidations permits only provisional and indefinite interpretations of the nature of the effects of environmental agents and conditions on the process.

Warburg(17, 18, 19, 20) has developed a theory of biological oxidations in which small quantities of iron in unknown combination with nitrogen are assigned the rôle of catalyzers in cell respiration. The iron is said to unite with molecular oxygen to form higher oxides, which then react with the adsorbed oxidizable materials of the cell and are returned to lower oxides. According to Warburg(18), cyanide is a specific negative catalyzer of this reaction, because it converts the iron to a form that is incapable of transferring oxygen. The reaction between the cyanide and the iron is held to be stoiochemical, and to this Warburg ascribes the fact that the degree of depression of oxidations in the cell depends upon the concentration of cyanide. It is also held that the degree of depression is independent of the period of exposure to the cyanide, presumably because the quantity of iron within the cell is exceedingly minute and the end point of its reaction with the cyanide is reached very quickly. This theory, while attractive, does not, in its present form, accord with the known facts of the effect of cyanide on biological oxidations. In the first place, there is some evidence that appropriately diluted solutions of KCN accelerate oxygen consumption in living organisms(21, 2). Nor is it possible to obliterate oxygen consumption completely by means of cyanide. Cyanide is not, therefore, a specific negative catalyzer in the strict sense that Warburg's theory requires. Secondly, the recovery of oxidations in the presence of cyanide is due, according to Warburg, to the oxidation of the cyanide itself by the catalytic action of the iron. Since the theory holds that the cyanide renders the catalyst incapable of transferring oxygen, a reasonable doubt arises that a catalytic agent that has thus been inactivated can oxidize the substance that has rendered it incapable of transferring oxygen.

Thirdly, any workable theory of the action of cyanide on cell respiration necessitates accounting for the rise of oxygen consumption to a rate above the normal during recovery. To bring Warburg's conception into alignment with this fact, one must assume an increase in the quantity of active iron or of oxidizable substances in the cell during the period of lowered oxidative activity. Since the extent of the increase in oxygen consumption above the normal is independent of the duration of depression, these two assumptions may be regarded as unlikely. A third assumption is possible, namely, that the catalyst, on recovery from the cyanide effect, becomes excessively active. Concerning this possibility there is no information available. One must conclude that Warburg's theory in its present form does not satisfactorily account for the results reported here.

The data show clearly that not only is the recovery of oxidative metabolism rapid, but also that the rate of oxygen consumption rises appreciably higher than the normal within one hour after removal from the KCN. This occurs in eighty-six cases out of ninety-two. Some of Child's(8, pp. 386, 388) and Allen's(9, pp. 112, 113) data are in agreement with these. Geppert's paper(1, p. 315) also contains data that are suggestive. A statement by Lund(22) in pointing out certain differences between the effect on oxygen consumption of KCN and deficient oxygen tension, that no such supernormal rate of oxygen consumption occurs after exposure to KCN, appears to overlook a fact that is indicated in Allen's data, to which he refers for his evidence on the effect of KCN.

In the present work attention has been confined almost exclusively to one concentration of KCN, namely, M/25,000. The six experiments with M/50,000 serve to indicate, 1) that depression in this concentration is not as great nor as rapid as in the stronger solution; 2) that a supernormal rate of oxygen consumption occurs after exposure to solutions more more dilute than M/25,000. It does not follow, however, that a supernormal rate should be expected after exposure to stronger solutions than M/25,000, and Child's(8) data indicate that under such conditions a supernormal rate of carbon-dioxide production does not occur.

It is of interest to contrast the results recorded here with those of Lund(22) on the effect of low oxygen tension on the oxygen consumption of Planaria agilis. Briefly stated, Lund shows that, in environments of deficient oxygen content, the rate of oxygen consumption decreases continuously up to the time limit of his experiments—fifty hours. When the animals are returned to air-saturated water, the rate of oxygen consumption rises rapidly from 2 to 80 per cent above the normal. Lund compares these results with those of Allen(9) on the effect of KCN on oxygen consumption, and concludes that the effects of low oxygen and cyanide are not identical. So far as can be determined from the literature, no modern investigator holds that the mechanism of the resulting depression of oxidative metabolism is identical for both. But Lund's results add little information as to the nature of the differences. They show that with decreasing oxygen content of the environment the rate of oxygen consumption decreases. Both Allen(9) and the writer have used a constant concentration of KCN throughout the duration of each experiment and have found that the oxygen consumption remains fairly constant at a subnormal level. It is at once obvious that the results of Lund's experiments on low oxygen content and those of Allen(9) and the writer are not comparable, since constant oxygen concentrations were not maintained by Lund.

Recent investigations by Crozier(23) strongly indicate that biological oxidations involve more than one type of catalyzed reaction. It is also known that physical state and permeability conditions likewise are of importance in oxidative metabolism. Cyanide and deficient oxygen may affect the same or different factors. A common result is a depression of the rate of oxygen consumption and a return to a supernormal rate for a time after the depressing conditions have been removed. Further evidence of an intimate relation between the two effects is found in the fact that cyanide hastens the processes of cytolysis and death that result from deficient oxygen tension(8).

On the basis of a considerable body of evidence, Child has advanced the conception that the susceptibility of protoplasm to depressant agents or conditions is related to its rate of metabolism, i.e., within certain limits and over an appropriate range of concentrations or intensities, the higher the original metabolic rate the greater the depression produced. A review of the evidence for this conception of differential susceptibility is not required here, since adequate recent reviews appear elsewhere(24). Quantitative measurements of depression in relation to original rate of metabolism are few in number. At least two methods of approach are possible. One is to measure the effects of the same concentration of a depressant on two regions of an organism that are known to have different rates of metabolism. This Galigher(12) has done, showing that the oxygen consumption of anterior and posterior pieces of annelids, which have a higher rate of oxidations than middle pieces, is relatively more depressed by KCN. Another method is to take advantage of the fact that the normal rate of metabolism of certain animals varies from time to time, the variation being due to differences in state of nutrition, differences in age, and other causes. The determination of the effect of the same concentration of a depressant on the rate of oxygen consumption over a considerable range of normal variation should, if a sufficient number of tests are made, afford a mathematical test of the validity of Child's conclusion. It is believed that the eighty-six experiments on the effect of M/25,000 KCN given in the tables fulfill the conditions of a fair test, although a greater number of determinations and a wider range of variation from the mean are desirable. In calculating the coefficient the correlation between the original rate of oxygen consumption and the degree of depression, the writer has included only the experiments in the tables that give the oxygen consumption during the first two or four hours' exposure to M/25,000 KCN. Where there are determinations of both the first and the second two-hour periods, the lower of the two is taken as the depression figure. The calculations of the original rate of oxygen consumption are in terms of hundred thousandths of a cubic centimeter of oxygen consumed per milligram per two hours. The calculations of the depression are in terms of per cent of the normal, which is taken as 100 per cent in each case. On this basis the following are the results:

- Normal rate of oxygen consumption:
    - Extremes, 32, 63.
    - Mean, 46.3 ± 0.4052.
    - Standard deviation, 5.72 ± 0.2972.
- Depression:
    - Extremes, 27, 83.
    - Mean, 57.8 ± 0.5271.
    - Standard deviation, 7.25 ± 0.3730.
- Coefficient of correlation, 0.3775 ± 0.0673.

It is evident, therefore, that a correlation of appreciable magnitude between the rapidity of oxidations and susceptibility to KCN does exist, and that with increasing rate of oxygen consumption a relatively greater depression is produced.

A second conclusion that Child has drawn is that if depression has not exceeded certain limits, those regions of an organism, or organisms in which the original rate of metabolism is highest, recover more rapidly and more completely within a given time. If, however, depression has exceeded these limits, the regions or organisms of lowest original rate recover more quickly. If, in the present work, we regard the amount of oxygen consumed during the first hour after removal from the cyanide as evidence of recovery, some indication of this differential recovery might be expected to appear in a mathematical treatment of the data. Certain qualifications of this expectation must be made, however. In Child's evidence in support of his conclusions there is no way of detecting, except by the end results of recovery, the critical point of depression, below which regions or organisms of originally relatively higher rate of metabolism recover more slowly than those of relatively lower original rate. Similarly, in these results there is at present no method of detecting in which experiments this critical point of depression has been passed. Consequently, in including all the experiments on recovery in the calculations, it is extremely probable that both types of recovery are included, and that the calculations therefore represent the algebraic sum of the data on animals in which depression has exceeded the critical limit and on animals in which it has not been reached. Therefore, the calculated correlation is not to be considered decisive.

There are but sixty-three comparable experiments on recovery during the first hour from a four-hour exposure to KCN given in the tables. The variation in per cent of the normal rate of the amount of oxygen consumed during the first hour of recovery is very wide. The calculated results are:

Normal rate of oxygen consumption:
    Extremes, 32, 63.
    Mean, 47.9 ± 0.4875.
    Standard deviation, 5.74 ± 0.3450.
Recovery during first hour:
    Extremes, 72, 191.
    Mean, 125.9 ± 1.964.
    Standard deviation, 23.12 ± 1.389.
Coefficient of correlation, 0.1871 ± 0.0820.

They may be said to indicate only the possibility of a correlation between original normal rate and recovery.

A number of investigators, e.g., Collip(25), Krogh(26), have regarded the excess oxygen consumed on return of a facultative anaerobe to aerobic conditions as, 1) utilized in the reconstitution of a residual substance that functions as an intramolecular source of oxygen or, 2) in the oxidation of oxidizable substances which accumulate as products of anaerobic metabolism. While there is considerable evidence of the formation of such substances that is not disputed, the data in this paper indicate that the fact that an excess of oxygen is consumed after a period of depression does not necessarily constitute valid evidence of the reconstitution of a residual substance containing oxygen, nor of the presence of substances produced during anaerobic metabolism that are capable of being oxidized only by free oxygen. As has been pointed out, the extent of increase in oxygen consumption above the normal is independent of the duration of depression. It has also been shown by Collip(25) that the carbondioxide production during anaerobic existence is depressed by KCN (see also Fiecther,27), yet in Planaria, at least, excess oxygen is consumed when the animals are removed from, and are recovering from, its effects.

SUMMARY

Data on the effect of dilute solutions of KCN on the oxygen consumption of Planaria dorotocephala are given, including in all ninety-two experiments. Exposures to the cyanide range from two to twenty-four hours. Data on recovery in water up to the eighth hour after removal from the cyanide are also recorded.

In experiments in which the duration of exposure was four hours, the oxygen consumption fell rapidly to a certain level, where it remained without material change. On removal from the cyanide, the oxygen consumption rose to a point considerably above the normal, returning downward to the normal in six hours. In experiments in which the exposure to cyanide extended over twenty-four hours, the rate of oxygen consumption fell, as in the four-hour experiments, to a certain level where it remained without material change throughout the exposure. On recovery, the rate rose to a supernormal level. The extent of rise above the normal was independent of the duration of exposure to the cyanide.

The results suggest the possibility of the depressive action of KCN on oxidative metabolism being largely physical in nature. Warburg's theory does not offer an adequate explanation.

The coefficient of correlation between the original normal rate of oxygen consumption and the degree of depression in these experiments is 0.3775 ± 0.0673, which demonstrates within reasonable certainty that a correlation of appreciable magnitude between degree of depression and normal rate of oxygen consumption exists and substantiates the conclusion of Child regarding the basis of differential susceptibility.

The coefficient of correlation between the normal rate of oxygen consumption and the amount of oxygen consumed during the first hour after return to water is 0.1871 ± 0.0820. This indecisive value appears to be due to the probability that the calculations include experiments of two types, viz., those in which the depression has proceeded to the extent that recovery is relatively slow and those in which the critical point of depression, below which recovery is slow, has not been reached; recovery in these cases may be expected to be relatively rapid.

Since the extent of rise in rate of oxygen consumption above the normal during recovery is independent of the duration of depression, the fact an excess of oxygen is consumed after a period of depression does not necessarily constitute valid evidence of the reconstitution of a residual substance containing oxygen, nor of the accumulation of oxidizable substances during depression.

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SOME ANTAGONISTIC AND ADDITIVE EFFECTS OF ANESTHETICS AND POTASSIUM CYANIDE

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FOUR FIGURES

INTRODUCTION

Recognition of the importance of oxidations in vital phenomena has increased since the time of Lavosier, and their rôle in the elaboration of certain constituents of protoplasm, as a source of energy in the organism and in other vital processes, has in many cases been definitely established. Oxidations constitute one of the reliable indices of metabolic activity. There is also abundant evidence that form and differentiation in an organism are conditioned in part at least upon the relative rapidity of the physiological activities of its several regions, with which processes oxidations are intimately associated. Ether, alcohol, and KCN have been widely used as experimental agents in teratogeny, in regeneration, and in other experimental work. It is, therefore, important that the effect of these substances and others so employed on biological oxidations should be studied rigorously.

In this paper attention is confined to the effects of ether-potassium cyanide and alcohol-potassium cyanide mixtures on the oxygen consumption in flatworms and their combined action in producing death and disintegration. No attempt is made to review the extensive literature on the biological effects of these substances. Adequate reviews of the literature on anesthetics will be found in Lillie's paper(1) and in Winterstein's recent book(2). The present status of the problem of the effect of KCN on biological phenomena is given in Hyman's paper(3).

THE PROBLEM

When the first zooid of Planaria dorotocephala is severed into three equal parts, the head being cut off and discarded, the rate of oxygen consumption of the pieces rises immediately. Posterior pieces are more stimulated by the cutting than are anterior pieces. This increased rate of oxygen consumption disappears within forty-eight hours(4). It was shown in a former paper(5) that ether applied in suitable concentrations at the time of cutting prevents or inhibits this stimulation. In a later paper(6) it was shown that when like concentrations of ether were applied to the pieces after forty-eight hours after section, when the stimulation had disappeared, no depression of oxygen consumption below the normal resulted. Instead, slight increases were noted.

Investigations by Allen(7), Child(8), Hyman(3), and the writer(9) have shown that KCN, even in extremely dilute solutions, profoundly depresses the rate of respiration in Planaria.

It has been found that both ether and KCN are effective in increasing the number of heads regenerated in a given lot of pieces approximately the posterior thirds of the first zooid of P. dorotocephala when applied in appropriate concentrations at the time of cutting(10, 11). Under the same conditions, both ether and KCN bring about decreases in the head frequency of anterior thirds, but KCN is more effective than ether. When applied forty-eight hours after section, after the stimulation of cutting has subsided, KCN produces increases in head frequency in posterior pieces (unpublished data), while ether is almost totally ineffective(6). Both lower head frequency in anterior pieces.

Interpretations of these effects of ether and KCN on the regeneration of heads in pieces of Planaria have been made on the basis of the differences in their depressive effect on the relative rates of physiological reactions in pieces from different levels and in different regions of the isolated piece, which will be found in full in the papers mentioned. It is not within the province of the present paper to enter further into a review of the methods by which head frequency in pieces of Planaria have been controlled. The facts outlined show that, as regards effects on both oxidative metabolism and regeneration in P. dorotocephala, ether and KCN have certain similarities and likewise certain dissimilarities. It is proposed to present in this paper the results of experiments designed to test whether the effects of these substances on oxidative metabolism and in disintegration are additive or antagonistic. For reasons that will be apparent later, in one series of experiments ethyl alcohol was substituted for ether. A subsequent paper will deal with the effects of combinations of ether and cyanide on the head frequency of pieces of Planaria.

METHODS AND DATA

Oxygen consumption

The form used in these experiments was the fresh-water flatworm, Planaria dorotocephala, obtained from the University of Chicago laboratory and kept under the conditions which have been employed in that laboratory to maintain the animals in normal condition. Although this form is not ideal material for the study of the effects of ether on oxygen consumption, its employment in this work was necessary because it was desired to associate studies on regeneration with an examination of other biological effects of ether and KCN.

While the comparative effects of ether and KCN, and mixtures of the two, on animals of as nearly as possible identical physiological condition were desired, it was found inconvenient and almost impossible to measure the oxygen consumption of separate lots of animals in water, in an ether solution, in a cyanide solution, and in a mixture of ether and KCN simultaneously, and continue the experiment over a sufficient duration of exposure and recovery. Accordingly, the effect of ether acting alone was first tested.

The method of procedure was essentially as follows: Lots of forty or more animals of approximately the same length were selected from the same stock pan, and their oxygen consumption in tap-water was taken over a convenient period, two to sixteen hours. The water was then replaced by a freshly made tap-water solution of ether, and the oxygen consumption during the first two hours in the ether solution was measured. The solution was then replaced by fresh, and the oxygen consumption during the next two hours or a later two-hour period was taken, the solution of ether being renewed every two hours. Since all the air was excluded from the flasks containing the animals during these tests, loss of ether by evaporation was negligible. The animals and the containers were then washed in four or five changes of water for eighteen to thirty minutes, and the oxygen consumption in water during the first hour following was measured. The animals were then weighed, following the method given in a former paper(11). The Winkler method was used in obtaining the oxygen content of the water and solutions. A discussion of its reliability as employed in these methods will be found elsewhere(3, 11, 9).

In measuring the oxygen consumption of the animals in the solutions containing ether a serious difficulty was encountered, due to the fact that on admission of ether in any appreciable concentration, the animals, although immobilized by precautions noted elsewhere (p. 312), writhe for a considerable time. This motor activity, utilizing oxygen, tends to mask the effects of the ether or the ether-cyanide mixture on the basic oxidative metabolism. The difficulty was only partially avoided in the $\frac{1}{4}$ per cent ether solutions, but nearly so in $\frac{1}{2}$ per cent and $\frac{3}{4}$ per cent, by the following procedure: After the test of the oxygen consumption of the animals in water had been made, a solution of ether of the same concentration as that to be used in the oxygen-consumption test was introduced into the flasks containing the animals and allowed to stand for thirty minutes. At the end of this time, the writhing had visibly decreased and the flasks were then prepared for the oxygen-consumption test. Writhing continues to some extent during the following two hours, but if the animals are undisturbed either by moving the flasks or the advent of strong light, it almost totally disappears in the ½ per cent and ¾ per cent solutions at the end of this time. During the period from the fourth to the sixth hour of exposure to ¾ per cent ether, the animals are practically inert if undisturbed. It will be noted in the data in table 1 that the oxygen consumed during this period is as great, and in many cases greater, than during the earlier periods of exposure. If one will subject these animals to ¾ per cent ether for four hours and then observe their behavior while undisturbed during the succeeding two hours in the solution, he will be convinced that motor activity is very slight and insufficient to account for any differences in oxygen consumption between animals so treated and controls. It seems that the results recorded throughout the exposure to ether solutions are but slightly or not at all influenced by motor activity.

The concentrations of ether employed were ¼ per cent, ½ per cent, ¾ per cent, and in a few cases 1 per cent. At room temperature the animals die in 1 per cent ether if the exposure is continued beyond three hours. Accordingly, weaker solutions were used. Under ordinary conditions of temperature and nutrition, animals of moderate size recover from a six-hour exposure to ¾ per cent ether completely in less than twelve hours. Total anesthesia with ether does not appear to be possible in P. dorotocephala; even in 1 per cent ether the animals respond to stimuli after the exposure is extended beyond the time within which complete recovery is possible. As shown above, anesthesia in ½ per cent and ¾ per cent ether is not total even after long exposure.

The KCN concentration M/25,000 was selected because this strength depresses oxygen consumption more than 50 per cent(3, 9).

In the experiments with combinations of ether and KCN and with KCN alone, the same procedure was followed, additional lots of animals in the desired KCN solution being tested simultaneously with those in the ether-cyanide mixture.

Other technical precautions were observed as follows: Temperature: The temperature at which each test was made was maintained within less than one degree by immersing the flasks containing the animals in a water-bath during the test. Motor activity: This was reduced to a minimum by introducing the animals into the flasks some hours before the start of the experiment, by having the temperature of the experiment slightly below that to which the animals were accustomed, by excluding light during the tests, and in many cases by beheading the animals. Hydrogen-ion concentration: Tap-water freshly drawn for these experiments has a hydrogen-ion concentration of pH 7.0 to pH 7.2 (phenol-red method). After standing for twelve hours, the alkalinity increases to pH 7.4 to pH 7.6. No differences in oxygen consumption of animals in freshly drawn tap-water when compared with that of animals in water that had stood for twelve hours or longer was noted. The addition of ether in the concentrations employed does not appreciably affect the hydrogen-ion concentration of the water. The addition of M/25,000 KCN, however, increases the alkalinity slightly, pH 0.4. A similar change of pH in tap-water being without appreciable effect on the rate of oxygen consumption, it was considered unnecessary to bring all solutions to the same pH value. The effect of KCN on the titration involved in the Winkler method has been discussed elsewhere(3, 9). Ether does not affect the Winkler reactions, nor do ether and KCN react with each other.

Data

Table 1 gives the complete data obtained on the effect of ether on the rate of oxygen consumption. Six experiments appear in a previous paper(6) with substantially the same results. In all, seventy-four determinations of the oxygen consumed in ether are given here, of which number sixty are greater than the comparable determinations of the oxygen consumed in water. Of the fourteen exceptions, six are found among the ten determinations of the effect of $\frac{1}{4}$ per cent ether, and only five show distinct depression of the oxygen consumption in ether. The conclusion is warranted that ether in these concentrations tends to increase oxidative activity in P. dorotocephala, the increase being greater in ¾ per cent than in lesser concentrations. In general, there is a distinct drop below the normal rate of oxygen consumption during the first hour of recovery from the effects of ¾ per cent to dilute KCN(9). On exposure to M/25,000 KCN the oxygen

TABLE 1

Effect of ether on the oxygen consumption of Planaria dorotocephala. In 0.00001 cc. per milligram per two hours. In water over two to sixteen hours, four to six hours in ether, and first hour after return to water. Beheaded animals. Bracketed data are experiments run parallel. Twenty degrees, except last four at 22°

LENGTH	TIME SINCE FEEDING	WEIGHT	CONCENTRATED ETHER	OXYGEN CONSUMPTION IN WATER	ETHER			WATER
					First two hours	Second two hours	Third two hours	First hour
mm.	*days*		*Per cent*					
18	3	535	¼	57	36	47		62
18	3	486	¼	36	35	41		56
18	4	494	¼	53	36	55		61
18	7	390	¼	50	50	50		49
18	1	502	¼	50	30	51		42
19	2	674	½	39	31	50		
19	2	665	½	42	54	44		
19	2	614	½	44	51	42		
19	2	679	½	40	34	21		
18	7	513	¾	44	67	70		
18	7	519	¾	42	67	62		
18	7	535	¾	42	63	50		
18	7	544	¾	40	50	28		
22	3	713	¾	38	59	54		
22	3	722	¾	39	52	46		
22	3	700	¾	40	53	51		
22	3	716	¾	38	43	33		
22	6	595	¾	42		58	57	44
22	6	602	¾	44		62	59	34
22	6	604	¾	41		57	46	23
22	6	552	¾	43		56	50	35
22	2	632	¾	41		53	74	32
22	2	609	¾	43		61	67	48
22	2	738	¾	40		50	58	38
22	2	568	¾	40		47	46	40
25	6	482	¾	47	91	73	77	42
25	6	497	¾	47	85	66	69	61
25	6	492	¾	46	79	67	69	51
25	6	487	¾	46	70	53	68	38
25	2	631	¾	55	77	70	74	25
25	2	685	¾	53	62	63	62	40
25	2	652	¾	54	63	53	78	32
25	2	637	¾	54	49	35	56	26

consumption of Planaria falls rapidly to a certain level, where it remains without material change for at least twenty-four hours. On return to water, it rises during the first half-hour to a supernormal rate, which persists for at least six hours. The contrast with ether is therefore sharp, the changes in rate of oxygen consumption being different in direction, both during the treatment and in recovery.

Graphs of the data on oxygen consumption in ether-cyanide solutions and in cyanide alone are given in figures 1, 2, and 3. They show that the combination of the two agents is slightly less effective in lowering oxygen consumption than is the cyanide alone; they also show that the protection afforded by the ether is greater during the second two hours of exposure than during the first. In the thirty-nine experiments from which these graphs are taken, twenty-nine of the lots in ether-cyanide show a higher average oxygen consumption per two hours than do the controls in cyanide, while ten are exceptions. Although the differences are not great and the exceptions are frequent, these results show that ether in these concentrations serves to protect to a slight extent against the depressive action of M/25,000 KCN on oxidative metabolism. The suggestion might be made that, in the light of the data on the effect of ether acting alone, given in table 1, this slightly higher rate of oxidations in the ether-cyanide mixtures may be due to the stimulatory effect of ether. But it has been shown that the higher the original rate of oxygen consumption, the greater the depression produced by this concentration of KCN (9, 12). It is, therefore, not only improbable that the stimulatory effect of the ether tends to mask the depressive effect of the KCN, but extremely probable that any greater susceptibility of the animals due to their slightly higher rate of oxidations is prevented by the protective action of the ether, since one would expect the animals with the higher rate of oxygen consumption to be more depressed by the cyanide. Consequently, one may rea-
ether. This is just the opposite of what occurs after exposure
sonably regard the protective action of the ether as greater than is evident in the data.

To test more thoroughly the possibility of a protective action of anesthetics against cyanide depression, a similar series of experiments in which ethyl alcohol replaced the ether was carried out. In order to eliminate any possible differences in motor activity between the animals in alcohol solutions and those in water, or water and KCN, the animals to be used were placed in appropriate alcohol solutions for several days and, in some cases, several weeks before the experiment. The concentrations used were non-narcotizing, except for a slight sluggishness during the first day of exposure; if compared with animals living in water after that time, no differences in behavior can be noticed(11). Table 2 gives the complete results obtained. Inspection of these data

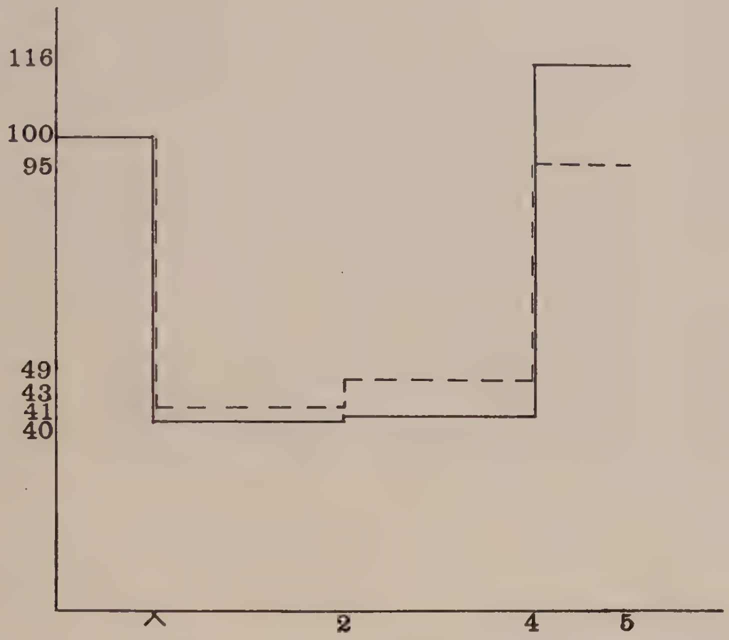

Fig. 1 Broken line, oxygen consumed per two hours per milligram in $\frac{1}{4}$ per cent ether-M/25,000 KCN mixture. Solid line, oxygen consumed in M/25,000 alone. Ordinates are oxygen consumption in per cent of the normal. Abscissae are time in hours. The period from the fourth to the fifth hour is that of recovery in water. Average of thirteen experiments.

reveals that alcohol exerts a protection against the action of M/25,000 KCN that is comparable in degree with that of ether in the concentrations employed. Out of twenty-six cases, twenty-three show the average oxygen consumption per two hours in the alcohol-cyanide mixture greater than in the cyanide alone. Remembering that animals of higher original rate may be expected to show more depression by KCN, it seems probable that, in the case of alcohol as well as ether, the protective action is greater than is apparent in the data.[1]

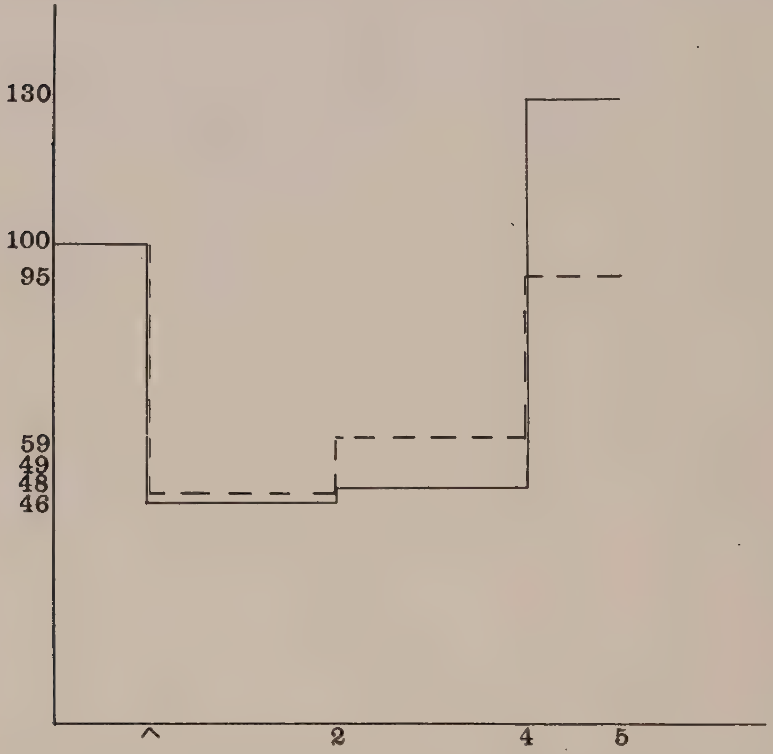

Fig. 2 Broken line, oxygen consumed per two hours per milligram in $\frac{1}{2}$ per cent ether-M/25,000 KCN mixture. Solid line, oxygen consumed in M/25,000 KCN alone. Ordinates are oxygen consumption in per cent of the normal. Abscissae are time in hours. The period from the fourth to the fifth hour is that of recovery in water. Average of fourteen experiments.

However, the possibility of a slight reduction in the concentration of the cyanide in the alcohol-cyanide mixture, due to interaction, must be taken into account, although it may be regarded as unlikely under the conditions of the experiments that any appreciable quantity of ethyl cyanide is formed.

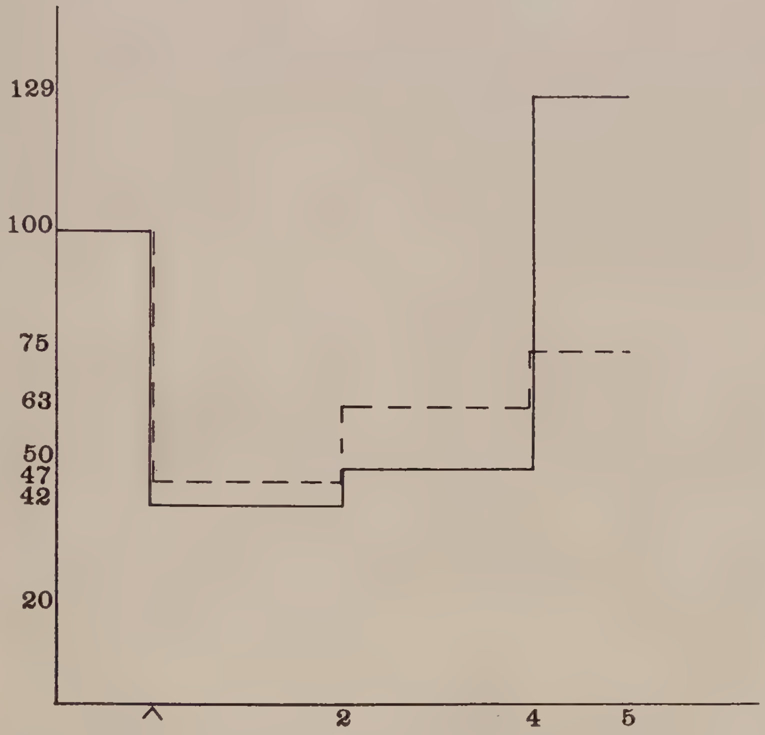

Fig. 3 Broken line, oxygen consumed per two hours per milligram in ¾ per cent ether-M/25,000 KCN mixture. Solid line, oxygen consumed in M/25,000 KCN alone. Ordinates are oxygen consumption in per cent of the normal. Abscissae are time in hours. The period from the fourth to the fifth hour is that of recovery in water. Average of twelve experiments.

Summing up, the data on the effects of combinations of ether and cyanide, and of similar combinations of ethyl alco-

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[1] While the rates of oxygen consumption of the animals living in alcohol solutions are distinctly higher than the controls, the differences are not as great as those given in a former paper (11).

hol and cyanide, show that these substances in certain concentrations have a slight protective action against the depressive effect on oxygen consumption of KCN solutions of the order of M/50,000 and M/25,000. The data given here and also those given in another paper(9) show that during the first hour of recovery from KCN the oxygen consumption of Planaria rises above the normal. Ether tends to prevent this increase in rate of oxygen consumption during recovery. Alcohol in the concentrations employed differs in this respect, the animals when returned from an alcohol-cyanide solution to an alcohol solution consuming as much or more oxygen during the first hour than the controls. This difference may be apparent only; stronger solutions of alcohol might give quite different results.

Disintegration

It was shown in an earlier paper(11) that animals after extended exposure to alcohol are much more susceptible to lethal concentrations of cyanide than are control animals. It is therefore evident that, while alcohol appears to act as a protective agent against dilute KCN, the effects of the two substances are in some way additive when the KCN is present in lethal concentrations. This fact suggests the question as to whether the effects of ether and lethal cyanide solutions are also additive.

A series of eight experiments on the susceptibility of the animals to a lethal concentration of KCN in an ether solution was carried out. The method, essentially that described by Child(12), consisted in selecting a lot of animals as nearly as possible the same length, appearance, and physiological condition. These were divided into five groups of ten each, each group being placed in separate small flasks. Treatment was as follows: Flask A was filled with a water solution of M/1000 KCN; flask B with a $\frac{1}{2}$ per cent ether solution of M/1000 KCN; flask C, after treating for two hours with $\frac{1}{2}$ per cent ether, was filled with $\frac{1}{2}$ per cent ether-M/1000 KCN; flask D, after four hours' treatment with $\frac{1}{2}$ per cent ether, was

TABLE 2

Oxygen consumption in alcohol plus KCN and in KCN alone. In 0.00001 cc. per milligram per two hours. Animals with heads. All experiments at 20°

LENGTH	TIME SINCE FEEDING	WEIGHT	TREATMENT WITH ALCOHOL		OXYGEN CONSUMPTION AT START	CONCENTRATION OF KCN	OXYGEN CONSUMPTION		WATER	
							First two hours	Second two hours	Average	First hour
mm.	*days*		*Per cent*	*days*						
20	7	340	Control		47	M/50,000	31	18	25	60
20	7	392	½	6	58	M/50,000	33	26	30	67
20	7	401	½	6	56	M/50,000	27	21	24	62
18	14	495	Control		47	M/50,000	39	40	40	64
18	14	384	½	14	69	M/50,000	35	33	34	64
18	14	360	½	14	76	M/50,000	41	31	36	86
18	14	636	Control		45	M/50,000	29	25	27	68
18	14	274	¾	14	87	M/50,000	47	53	50	90
18	14	276	¾	14	73	M/50,000	57	45	51	90
18	14	586	Control		44	M/50,000	23	24	24	67
18	14	302	¾	14	69	M/50,000	43	52	48	60
18	14	285	¾	14	61	M/50,000	49	53	51	60
18	7	619	Control		54	M/50,000	30	23	27	67
18	7	500	¾	7	70	M/50,000	39	19	29	63
18	7	462	¾	7	60	M/50,000	43	12	28	76
18	11	491	Control		42	M/50,000	26	31	29	62
18	11	524	¾	11	57	M/50,000	42	36	39	41
18	11	482	¾	11	63	M/50,000	30	42	36	60
18	7	490	Control		53	M/25,000	27	18	23	82
18	7	332	½	7	63	M/25,000	26	23	25	71
18	7	389	½	7	56	M/25,000	31	37	34	73
18	10	494	Control		50	M/25,000	26	20	23	76
18	10	438	½	10	63	M/25,000	38	29	34	66
18	10	556	½	10	56	M/25,000	31	37	34	66
18	21	515	Control		46	M/25,000	34	38	36	56
18	21	436	½	21	71	M/25,000	40	46	43	77
18	21	432	½	21	67	M/25,000	34	44	39	73
18	7	522	Control		49	M/25,000	29	31	30	70
18	7	324	½	7	80	M/25,000	36	34	35	97
18	7	345	½	7	76	M/25,000	36	32	34	105
18	21	637	Control		47	M/25,000	24	21	23	47
18	21	318	½	21	53	M/25,000	21	33	27	50
18	21	255	½	21	60	M/25,000	28	30	29	67
16	28	715	Control		39	M/25,000	21	21	21	55
16	28	270	½	28	67	M/25,000	22	28	25	58
16	28	337	½	28	62	M/25,000	21	26	24	70
18	11	554	Control		43	M/25,000	22	21	22	62
18	11	422	½	11	45	M/25,000	25	28	27	56
18	11	395	½	11	40	M/25,000	25	22	24	60

filled with $\frac{1}{2}$ per cent ether-M/1000 KCN; flask E, after a six-hour treatment with $\frac{1}{2}$ per cent ether, was washed out with water and then filled with a water solution of M/1000 KCN. The animals in each flask were examined hourly and their state of disintegration noted, the process of disintegration of each animal being recorded for convenience as stage I, stage II, etc., the criteria of Child being followed. Figure 4 is typical of the results. In plotting the data, it is necessary to assign numerical values to the proportion of the whole animal remaining alive at each stage of disintegration. Thus stage I is assigned the value 10, and since at the start of the experiment all of the animals in a flask are in stage I, the value for each flask at the start is 100 (plotted as ordinates). Stage II is assigned the value 8, and the combined value of the animals within a flask is reduced at the end of the first hour by the passage of some individuals into stage II, or even stage III. The determinations for succeeding hours are plotted in the same manner, stage III having the assigned value 6; stage IV, the value 4, and stage V, when disintegration is complete, the value 0.

Examination of figure 4 leaves no doubt that the presence of $\frac{1}{2}$ per cent ether hastens the processes of disintegration and death, although the animals tolerate this concentration of ether alone for twenty-four hours or more without any signs of disintegration. But with KCN present, the longer the exposure to the ether the more rapid the disintegration, whether the ether be present as in B, C, and D, or removed as in E. Whatever protective action ether may exert against dilute cyanide, the effects of the two agents are additive when the KCN is present in lethal concentration.

DISCUSSION

A considerable body of evidence shows that anesthetics, such as ether and alcohol, when present in moderate concentrations, decrease the permeability of living tissues. Thus, to mention only a few cases, Lillie(13) observed that certain concentrations of ether and other anesthetics decrease permeability. McClendon(14) has shown that electrical conductivity of sea-urchin eggs is decreased by chloroform, and Osterhout(15) has shown that ether and other anesthetics increase the electrical resistance of Laminaria, which are interpreted as evidence of decreased permeability. On the other hand, Krehan(16) has shown that KCN increases the permeability of plant cells to many substances. An important difference in the effects of anesthetics and KCN is also pointed out by Heilbrunn(17), who shows that ether in concentrations which anesthetize sea-urchin eggs decreases the viscosity of the protoplasm, while KCN in anesthetizing concentrations increases viscosity. We have, therefore, in the effects of these two types of substances when used in combination, the proper conditions for an antagonism, and, in fact, Krehan(16) has found that the presence of alcohol or chloroform prevents to a marked extent the increase in permeability of plant cells in a cyanide solution.

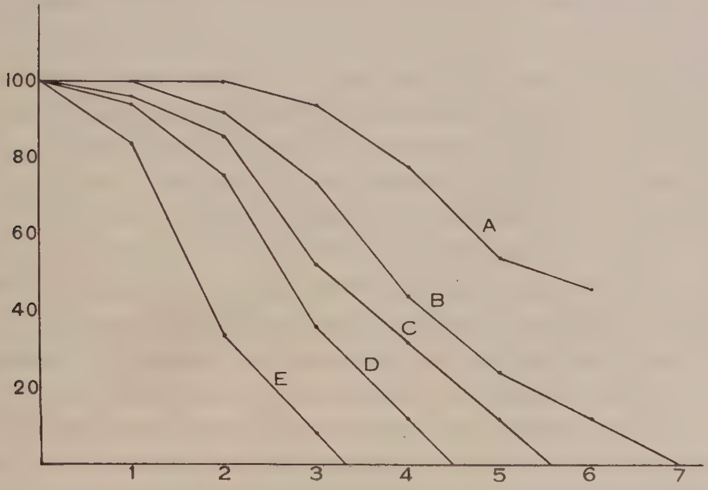

Fig. 4 The effect of treatment with ½ per cent ether on the rate of disintegration of Planaria in M/1000 KCN. *A,* disintegration in M/1000 KCN without treatment with ether; control. *B,* disintegration in M/1000 KCN made up in ½ per cent ether; *C,* disintegration in ½ per cent ether-M/1000 KCN after two hours' exposure to ½ per cent ether; *D,* like *C,* except that prior treatment with ether extended over four hours; *E,* disintegration in M/1000 KCN after six hours' exposure to ½ per cent ether and repeated washing in water.

In the light of these observations, it is not surprising that a certain antagonism is found in the effects of these substances on oxygen consumption in Planaria. The data on the relative rates of disintegration of animals by M/1000 KCN in the presence of ether or alcohol(11) show, instead of a protective, an acceleratory effect. We are then confronted with experimental evidence that one and the same concentration of an anesthetic in one case protects the organism against the action of KCN, and in another case increasingly augments its effect.

These results may with advantage be contrasted with those reported by others. Traube(19) found that anesthetics protect blood corpuscles against the action of a number of cytolytic agents; Arrhenius and Bubanovic(20) observed that weak solutions of anesthetics protect blood corpuscles against the cytolytic action of hypotonic solutions, while stronger solutions accelerate cytolysis; Osterhout (15) found that while moderate concentrations of anesthetics decrease permeability in Laminaria, as shown by an increase in electrical resistance, high concentrations bring about increased permeability and a cytolytic effect that is injurious and irreversible; Lillie(13) has made somewhat similar observations on the effect of ether and alcohols on permeability in Arenicola larvae, moderate concentrations decreasing and high concentrations increasing permeability and inducing cytolysis. Lillie regards the cytolytic effect as a consequence of increased permeability. The results reported here do not appear to be subject to this interpretation. Concentrations of anesthetics were employed which the animals will tolerate for a day or more, in the case of alcohol for weeks, without any evidence of disintegration and from which recovery is complete. In the experiments on disintegration, the concentration of the anesthetic remained unchanged, while a powerful cytolytic agent, M/1000 KCN, was added. To regard the protective action of the anesthetic as an evidence of decreased, and its acceleration of cytolysis as evidence of increased, permeability requires that the ether or alcohol simultaneously decreases and increases permeability to KCN—an obvious impossibility.

Warburg(21) has advanced a theory of biological oxidations in which small quantities of iron in unknown combination with nitrogen are assigned the rôle of catalyzers. The theory holds that molecular oxygen forms higher oxides of iron which then react with the substrate and are returned to lower oxides. Anesthetics interfere with oxidations because they are adsorbed on the surfaces of the catalyst and thus alter its ability to act. Cyanide interferes with oxidations by converting the iron to a form which is incapable of transferring oxygen. The anesthetics are, therefore, general, and cyanide specific negative cytalyzers. No provision is made in this theory for the fact that certain concentrations of many narcotic substances increase oxidative activity. It is conceivable, however, that the protective action of anesthetics may in part be due to the fact that their adsorption constants are in general much higher than that of cyanide. This would result in the displacement on surfaces of the more powerful depressant by one that is less effective in reducing oxidative activity. A more probable explanation of the results recorded in this paper is that the protective action of anesthetics against depression of oxidations by dilute KCN is evidence of decreased permeability, and their action in augmenting cytolysis by strong cyanide is evidence of a totally different effect. Both the protective and the acceleratory actions of the anesthetics on the KCN effects cannot be ascribed to permeability changes due to the presence of these substances. Evidence from many lines indicates that the protective effect is due to decreased permeability in the anesthetics, which results in lessening the internal concentration of the cyanide.[2] The facts suggest that these anesthetics, in addition to effects on permeability, bring about a sublethal or incipient state of disorganization which is reversible, but is unmasked by the cytolytic action of a strong cyanide solution, so that the injurious effects of the two substances become additive.[3] Increase in permeability may accompany the processes of disintegration, but some other factor is responsible. A similar explanation is indicated in some work of Child(8), which shows that lethal cyanide solutions and lack of oxygen when acting together produce death and disintegration in Planaria much more rapidly than does either acting alone.

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[2] Bodine(18) has shown that cyanide enters the cell undissociated and with considerable rapidity.

The question of the importance of the state of permeability in controlling the depressive effect on protoplasm of KCN and other agents is frequently raised. As regards the rôle of permeability in controlling the depressive action of KCN on oxidations in Planaria, a partial answer is given in these results. If these anesthetics, ether and ethyl alcohol, have any effect whatever on permeability in these animals, and in view of the evidence cited that anesthetics affect permeability in many types of living protoplasm—the assumption that they do is not unreasonable—this effect is evident in the oxygen-consumption data only as a slight lessening of the depressive action of the cyanide in the presence of these agents.

SUMMARY

Ether in concentrations of $\frac{1}{4}$ per cent, $\frac{1}{2}$ per cent, and $\frac{3}{4}$ per cent, and alcohol in concentrations of $\frac{1}{2}$ per cent and $\frac{3}{4}$ per cent, afford a slight protection against the depressive action of M/25,000 KCN on oxygen consumption in Planaria.

Ether in these concentrations hastens the processes of death and disintegration of the animals in M/1000 KCN.

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[3] In the progress of haemolysis of the red blood corpuscles of Spelerpes, Aggazzotti(22) has described with the ultra microscope a coagulation accompanying the initial effect of both hypotonic salt solutions and lipoid solvents. The question of the part played by hypotonicity in the disintegrative action of KCN on Planaria has recently been raised by Wilson(23).

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ON THE PRODUCTION OF DOUBLE LIMBS IN AMPHIBIANS[1]

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FORTY-SIX FIGURES

CONTENTS

Introduction 419
Material and methods 421
Experimental 423
  I. Reduplications produced by transplanting limb buds 423
    A. The development of the double limb 423
    B. Anatomy of the double limb 434
  II. Experiments in splitting the limb bud 440
    C. Rudiments split in stages 27–34 441
      1. Rudiments split dorsoventrally 445
      2. Rudiments split anteroposteriorly 448
      3. Rudiments split obliquely 449
      4. The limb girdle 452
    D. Limb buds split in stages 38–39 455
General discussion 459
  III. Relations of symmetry in spontaneous reduplications 460
  IV. Relations of symmetry in artificially divided limb buds 464
General summary and conclusions 468
Literature cited 470

INTRODUCTION

It has been known for a long time that extra appendages sometimes occur in nature, but their production by experimental methods dates back only to the last decade of the nineteenth century. Most of the well-controlled experimental work has been done on the amphibia, although the crustacea have been used to some extent.[2] The methods employed have varied from simple splitting of a regenerating or embryonic bud to the cutting and breaking technique used by Tornier and della Valle and the no less effective transplantations carried out by Braus, Harrison, and others.

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[1] This paper constitutes a revision of a dissertation presented for the degree of Doctor of Philosophy in Yale University.

[2] Some of the names associated with work on the experimental production of double limbs in amphibia are Barfurth ('94), Braus ('05, '09), della Valle('13), Detwiler ('18, '20, '22), Fritsch ('11), Giard ('95), Gräper ('22, '23, '24),

Before passing to a consideration of the experimental work which forms the basis for this paper, it may be well to consider briefly some of the general rules to which the reduplicated parts conform. The rules given by Bateson ('94) in his ''Materials for the Study of Variation'' have been modified by Harrison ('21) as follows:

''I. The long axes of duplex or multiplex appendages lie in one plane.

''II. Two adjacent members form, in structure and position, images of each other as reflected from a plane mirror bisecting the angle between the respective axes and perpendicular to the common plane of the two axes.''[3] These rules include not only the triplex appendages which were considered so fundamental by Bateson, but also the duplex, which have been shown by Harrison to be even more common under certain conditions.

Harrison ('07, '18, '21), Kurz ('12), Lissitzky ('10), Morrill ('21), Nicholas ('24), Piana ('94), and Tornier ('96, '97, '01, '05); and in the other groups, chiefly crustacea and insects, reduplication of appendages has been induced by Emmel ('07), Hogue ('15), Megusar ('07), Przibram ('02), Reed ('04), and Zeleny ('05). Excellent compendiums are to be found in the works of Bateson ('94) and Przibram ('21), while Pol ('13) gives a good brief review of the work up to that time.

[3] Practically all known cases of triplicate appendages conform to these rules. A supernumerary double chela in a lobster has recently been described by Dawson ('20) as an exception. In this case two extra chelae, both of which were crushers, were borne on a claw of the nipper type. These crushers were symmetrical with respect to each other (one right and one left), but the one nearer the normal was not mirrored with it. Since the primary chela of the triplicate appendage and the normal chela of the other side of the body were both of the nipper type, the case may be analogous in this respect to the two cases reported by Emmel ('06) in which two crusher claws regenerated after amputation of the normal symmetrical chelae. The case is also an exception to Przibram's ('11) rule which states that ''Supernumerary appendages with secondary symmetry in the nipper claw exhibit always the characters of nippers and even those with the symmetry of the opposite body-side form no part of the large, or 'crusher claw,'' (p. 257). Compare also Swett ('24).

In a study of limb reduplication, a number of points demand consideration. We find some cases which conform to the above rules and others which apparently violate them. The various types of extra members demand explanation both as to their morphology and their mirror relationships. There is a possibility that a plane of division of the embryonic or regenerating bud may be correlated with the mirror plane of a resultant reduplication, and, lastly, we are tempted to speculate on the ultimate causes of doubling. The present study contributes toward the solution of these questions and, while not professing to settle any of them definitely, points out possibilities which are instructive.

It gives me great pleasure to express my sincere gratitude to Prof. Ross G. Harrison for his active interest and advice during the course of this investigation.

MATERIAL AND METHODS

In starting this problem various procedures were used to divide regenerating limb buds of adult Diemyctylus viridescens[4] with the idea of ascertaining a possible relationship between the plane of division and the plane of symmetry of the resulting reduplication. The methods employed proved so inadequate and difficult to control that this phase of the problem was temporarily abandoned. The present communication is based on a large number of double limbs produced in the larvae of Amblystoma punctatum (Linn.) by various experimental methods.[5]

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[4] A considerable number of similar experiments were also made on fiddler crabs (Uca pugnax, and pugilator) of various sizes and on large hermit crabs (Pagurus pollicaris) with negative results. The animals were induced to autotomize appendages—walking legs being used chiefly—and the resultant stump was divided anteroposteriorly or dorsoventrally a few days after the autotomy.

[5] The recent work of Stockard ('21), in which he produced duplicities in Fundulus embryos by temporarily arresting the development of the eggs, suggested the possibility that double limbs might be produced in a similar fashion. Stockard found that by lowering the temperature or directly cutting off the oxygen supply at a certain stage of development, a certain percentage of double embryos resulted. One thousand Amblystoma embryos at various early stages

It was deemed advisable, in order that the function of the grafted limbs might be as perfect as possible, to begin the study of the problem on limbs situated in normal location. In this position a large number of reduplications can be made to develop if the anteroposterior axis of the limb disc be reversed. On the right side of the body two combinations bringing about this condition are possible, viz., 1) a right limb bud inverted (homopleural dorsoventral) and, 2) a left limb bud not inverted (heteropleural dorsodorsal). Both of these experiments were carried out in connection with the present study and a large number of double limbs, including a full set of developmental stages, secured.[6] Nearly all the limb buds were transplanted to the right side of the body, and, unless otherwise stated, all descriptions apply to this side.

In the routine experiments (homopleural dorsoventral) the dorsal margin of the disc was rotated over its anterior semicircumference slightly more than 180°. Part of the tendency to recover by rotation was thus obviated (Nicholas, '24) and the production of double buds made more certain. A considerable number of animals in this series was preserved before any limb had appeared for the purpose of studying the earlier stages in the process of reduplication.

Supernumerary limbs were also produced by artificial splitting of the limb rudiment. The two portions were kept apart by a barrier of indifferent tissue which was transplanted into the slit made in the disc (p. 440).

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in the development of the limb were submitted to a variety of abnormal conditions designed to inhibit metabolic activity. Different series were exposed during twenty-four- and forty-eight-hour periods to a variety of concentrations of alcohol, magnesium sulphate, chloral hydrate, and acetone, and in one series the temperature was lowered by placing the dish containing the embryos on a cake of ice. Development was inhibited by cold, but proceeded normally after removal of the embryos to room temperature. Non-toxic concentrations of the solutions used in all cases permitted normal development after their return to water. No specific abnormalities of the limbs were observed in any case.

[6] In addition to the material resulting from my own operations, there has been available Professor Harrison's collection of double-limbed Amblystoma larvae. I am indebted to him for permission to use this collection and the notes which record the histories of the individual cases.

Individual protocols were kept and the steps in the development of the limbs recorded at intervals of from one to four days. In particularly interesting cases periodic camera-lucida drawings were made.

EXPERIMENTAL

I. Reduplications produced by transplanting limb buds

Table 1 summarizes the results of transplanting limb buds with their anteroposterior axes reversed. Inasmuch as this axis is the only one the polarity of which is irreversibly determined at the time of operation (Harrison, '25), the first, or primary, limb to make its appearance is, as a rule, a limb of the opposite side of the body (disharmonic). This simple condition seldom remains long uncomplicated, however, because a supernumerary bud nearly always appears in some relation to the primary.

TABLE 1

Summary of experiments in transplanting limb buds

OPERATION	NUMBER OF EXPERIMENTS		SINGLE LIMBS DISHARMONIC		SINGLE LIMBS HARMONIC[1]		REDUPLICATED	
	Total	Positive	Number	Per cent	Number	Per cent	Number	Per cent
Hom. d. v.	220	146	5	3.42	9[2]	6.16	132[3]	90.41
Het. d. d.	54	32	3	9.37	0	0.00	29[4]	90.625

[1] By rotation.

[2] Including three cases of hyperdactyly.

[3] Including thirty-one cases in which single harmonic limbs resulted from reduplication with subsequent resorption of the primary limbs, also two cases of hyperdactyly.

[4] Including three cases of single harmonic limbs produced by reduplication and resorption of the disharmonic limb.

A. The development of the double limb. As contrasted with the normal,[7] the reduplicating limb bud is massive when it first appears, and has a slightly flattened, rounded top which faces laterally (fig. 1, *TR*). A slight constriction may appear at this time and divide the bud in one direction or another, thus imparting to it a bilateral symmetry. Anterior to this constriction a left limb grows out; posteriorly, a right is formed. This early division is not always noted, however, so that usually the first indication of what is going to happen is given by one portion of the bud rounding off, assuming a greater convexity on its anterodorsal border than over the rest of its surface, and starting to grow out from the body of the animal in a direction nearly opposite to that of the normal when viewed from the side (fig. 2, *PR*). Either synchronous with this primary limb or shortly after its appearance, another bud (fig. 2, *DU*) becomes distinct in the region posterior to the base of the first. This is the secondary limb, or the reduplicant, and is harmonic. The mirror plane between the two limbs so formed is radial. The typical subsequent development of these buds reveals nothing not shown

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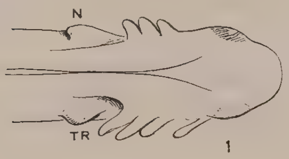
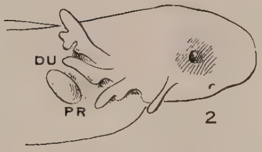
[7] Considerations of the normal development of the fore limb of Amblystoma may be found in the work of Clarke ('79), Harrison ('18), and Swett ('23). In addition, the accounts of Strasser ('79), Goette ('79), and Rabl ('01) are valuable for establishing points of comparison with other urodeles.





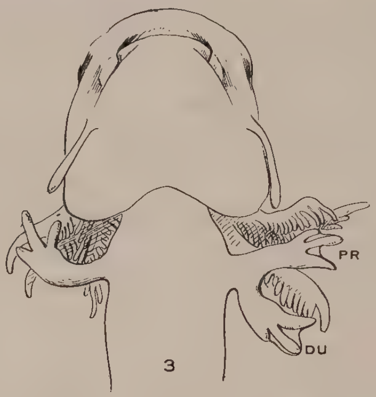


Fig. 1 Experiment Hom. d. v. 53. Right limb rudiment inverted. *TR* is a typical reduplicating bud. Stage 37, four days after operation. × 11.

Fig. 2 Experiment Hom. d. v. 84. Right limb rudiment inverted. Primary bud pointing in opposite direction from normal, secondary assuming normal position. Stage 39, six days after operation. × 11.

Fig. 3 Experiment Het.d.d.11. Right limb rudiment to left side, dorsodorsal orientation. Resulting limbs nearly perfect, mirror plane radial. Seventeen days after operation.

in the development of the normal single limb. The result is a pair of perfect limbs, right and left, completely separate from each other and normal in form and function. The specimen shown in figure 3 approaches this ideal arrangement, but most cases are far from being so simple as this.

Inasmuch as the fundamental changes leading to the production of double limbs are already instituted when the bud first becomes visible externally, serial sections of a number of stages ranging from embryos killed the day after operation to larvae with well-developed reduplicants were examined. Mitosis counts made in both normal and transplanted buds (table 2) showed that there are no apparent differences between them on the day after operation. Furthermore, no definite aggregations of cells or mitoses can be made out until the bud is divided into two parts which are visible externally. A slight initial retardation of mitotic activity is evidenced by the fact that, just subsequent to division, the two members of the complex may contain fewer mitoses than

TABLE 2

Showing the result of mitosis counts in limb buds of different degrees of doubling

NUMBER OF OPERATION	STAGE OF LIMB		CONDITION OF RIGHT LIMB (EXTERNAL)	MITOSES			
	Right (operated)	Left (normal)		Right (operated)			Left (normal). Total
				Ventral (anterior)	Dorsal (posterior)	Total	
Hom. d. v.							
132	34	34	Not visible			10	11
131	34	34	Not visible			15	16
121	36	36	Not visible	13	4	17	8
123	35	35	Not visible	17	10	27	18
194	37+	37	Disharmonic			69	74
83	Large	39	T. R. B.[1]			37	17
35	38+	39	T. R. B.	37	20	57	
197	37−	38	T. R. B.	31	26	57	53
191	37?	37	Two buds	45	6	51	72
173	37?	38	Two buds	28	58	96	114
199	Large	37	Two buds	81	45	126	83
116	40–41	42	Two limbs	147	160	307	

[1] Typical reduplicating bud.

the normal limb. This condition prevails for only a short time, however, for as soon as the double limb becomes adjusted to its environment, each of its components may rapidly overtake the normal in development. There is no distinguishable arrangement of mitosis planes in any of the buds, the cells apparently dividing in any direction with reference to the axis of the limb and the division plane of the reduplicating bud. This study, then, affords no evidence concerning the causes of doubling. These must be relegated to those invisible functions of the cells which are for the present beyond the range of observation.

For lack of space we may only name at this time the extensive fusions, rotations, reductions, and further reduplications which often occur and which Harrison has already mentioned as complicating the processes of development to a marked degree. These fortuitous factors may hopelessly confuse the picture or, under some conditions, may serve to clarify it. In this connection we may examine with considerable care the phenomenon of rotation and its bearing on the symmetry relations between members of certain double limb complexes to be described below.

The phenomenon of recovery of posture by rotation was first observed by Harrison in following the development of some of the inverted (homopleural dorsoventral) limbs in his series and has been the subject of further study by Nicholas ('24).[8]

Occasionally a primary limb derived from an inverted rudiment will attempt to recover normal posture by rotation even when a supernumerary member is present. Three instances of such a process are commented upon below. When the significance of these somewhat exceptional cases is understood, we are enabled to see very clearly that the anteroposterior axis of the reduplicant is determined at the time of its formation and is polarized in a direction opposite that of its mate (i.e., the anteroposterior axis of the limb from which the reduplicant is formed), and that the dorsoventral axes of both the primary limb and its reduplicant are determined at a later stage of development by the tissue immediately surrounding their respective bases (compare Nicholas, '22).[9]

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[8] I am indebted to Doctor Nicholas for permission to use the manuscript of this work before it was published.

The first case we shall consider (fig. 4) is interesting because it shows to what extent the determination of the dorsoventral axis of a reduplicant may be affected by the tissues of the primary limb. This limb complex is composed of three members, the most caudal (*DU′*) being very nearly normal, while the two anterior (*PR–DU*) are closely fused, their palms face dorsomedially and the complex projects dorsally.

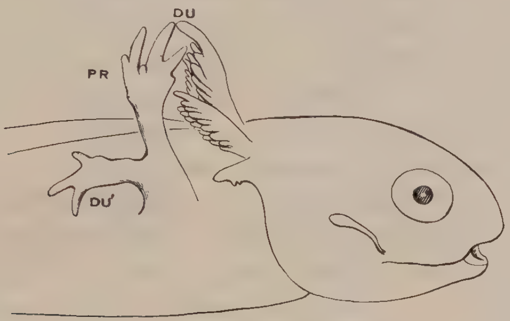

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Fig. 4 Experiment Hom. d. v. 9. Right limb rudiment inverted. Primary (*PR*) and secondary (*DU*) limb in abnormal posture, mirror plane radial. The primary limb again reduplicated, forming a harmonic tertiary functional member (*DU′*). Thirty days after operation. × 11.

Had the primary limb succeeded in the attempt which it made to recover its normal posture by rotation early in development, it would have been a right, right side up; since the rotation was incomplete, it is a left, upside down. Its posture is no doubt partly due to the mechanical interference of the reduplicant (*DU*). This secondary limb is an inverted right, inverted because the primary limb (i.e., the tissue surrounding its base) is inverted, and a right in consequence of this determination of its dorsoventral axis and the fact that its radio-ulnar axis is mirror-imaged with relation to the corresponding axis of the primary limb. This case shows very strikingly how, on the one hand, rotation and mechanical interference by a reduplicant can throw a limb completely out of posture and, on the other, how such an inverted limb can produce a mirror-image mate. This case takes on an added significance when we compare it with the next one.

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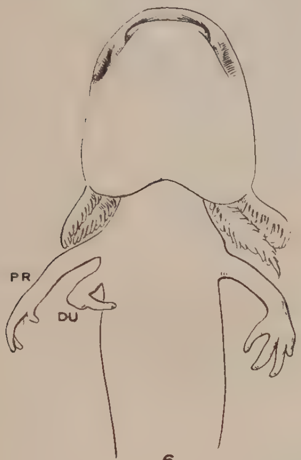
[9] By using a double operation, Nicholas watched the development of the limb in Amblystoma larvae which had had the area surrounding the limb rudiment rotated while the limb rudiment itself remained normally placed with reference to the body of the embryo. He found that rotation of the limb occurred with reference to the surrounding area, and not to the organism as a whole.

The case shown in figure 5 consists of two right limbs fused along their ulnar borders and merits a rather complete description. The history of the case (Hom.d.v. 15) shows good healing of the transplant with the first appearance of the primary limb (*PR*) on the fourth day after operation. On the next day, the bud was seen to point in an anterodorsal direction when viewed from the side. Six days later, the limb had practically recovered and was directed dorsolaterally and a little posteriorly. After eleven more days, its posture was practically normal for a harmonic member, although, because of the lack of power of supination, the palmar surface was directed dorsally when in repose. At this time an anterior (ulnar) reduplicant with two digits was noted and the upper arm was short and thick. At twenty-seven days after operation, the anterior limb was described as a right with three digits and the posterior (primary) member as a right apparently upside down. Two days later, the specimen was preserved. A study of serial transverse sections substantiated the diagnosis of palmar and dorsal surfaces—the palm of the anterior limb is ventral, that of the posterior is dorsal. The former (*DU*) is thus a right right side up and the latter (*PR*) a right upside down. These limbs are united by their ulnar borders with the palm of the anterior partly facing the dorsum of the posterior.

This remarkable case is interpreted as follows. The primary member is a right instead of a left, having undergone sufficient rotation during its early development to retain its original prospective asymmetry. Its inverted posture is attributable solely to its marked pronation and to the mechanical interference of the reduplicant. The extra member (*DU*) appeared on the ulnar border of the primary limb, and even though the two are closely fused, the former came sufficiently under the influence of the body of the animal to permit the usual determination of its dorsoventral axis, i.e., determination uninfluenced by the primary limb. Therefore, these limbs are of the same asymmetry, though their radio-ulnar axes are oppositely oriented. There is a possibility that the secondary member may be a regenerate from the host, but there is no evidence for this interpretation.



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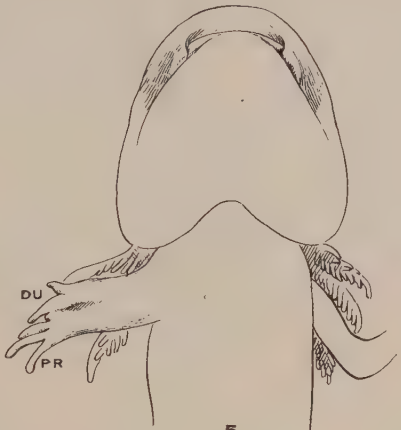

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Fig. 5 Experiment Hom. d. v. 15. Right limb rudiment inverted. The two limbs are united by their ulnar borders with the palm of the anterior (*DU*) partly facing the dorsum of the posterior (*PR*). Twenty-nine days after operation. × 11.

Fig. 6 Experiment Hom. d. v. 46. Right limb rudiment inverted. Both limbs are rights, the posterior (*DU*) somewhat abnormal. Forty days after operation. × 11.

Figure 6 also shows a specimen with two right limbs, but in this case the reduplicant became sufficiently separated from the primary in development to permit the latter to make a complete rotational recovery. The very early appearance of the reduplicant (*DU*) in this case, i.e., before rotation of the primary limb had started, explains why it is a right limb, differing in no respect from secondary limbs developing posterior to the usual disharmonic primary limb of the inverted bud (fig. 3). It is obvious from these cases that a limb cannot

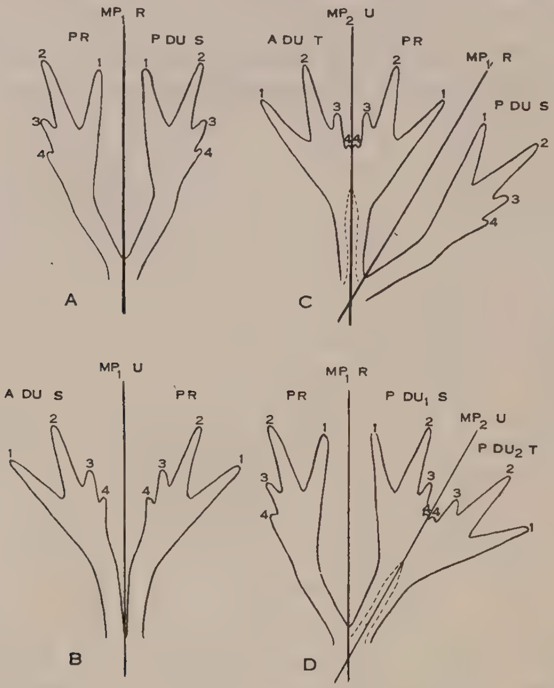


Fig. 7 Diagrams to show relations of symmetry in duplex and triplex appendages. (Modified from Harrison.) In these figures the radio-ulnar axes lie in the same plane, in actuality they are more often somewhat inclined. Abbreviations: MP_1, mirror plane between primary and secondary limbs; MP_2, mirror plane between tertiary limb and its image; *R*, radial; *U*, ulnar; *S*, secondary limb; *T*, tertiary limb; *PR*, primary limb; *A DU*, anterior reduplicant; *P DU*, posterior reduplicant.

A. Primary and secondary limbs mirrored in the radial plane. Most of the simple duplicities are of this type (fig. 3).

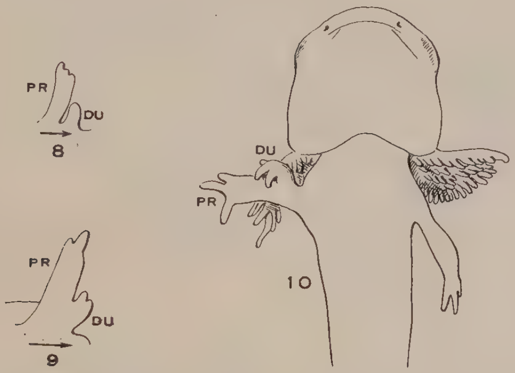
B. Primary and secondary limbs mirrored in the ulnar plane. A more unusual, but by no means a rare occurrence (fig. 10).

C. Primary and secondary limbs mirrored in the radial plane, tertiary limb the image of the primary. This is the usual arrangement between components of a triplex appendage.

D. Primary and secondary limbs mirrored in the radial plane, tertiary limb is the ulnar image of the secondary. The most unusual of the four types (fig. 13).

be called a right or a left until the polarity of both the anteroposterior and the dorsoventral axes is determined. The former is already determined by some unknown factor long before the stage used for operation (Detwiler, '18) and the latter is not until a later time,[10] and then strictly under the influence of the tissue surrounding its base.

The relations between the components of double and triple limbs are seen and sufficiently explained in figure 7. Aside from the general relations, which have been fully treated by Bateson ('94) and more recently by Harrison ('21), we may



Figs. 8 to 10 Experiment Het. d. d. 34. Left limb to right side, dorsodorsal orientation. Primary limb (*PR*) formed an anterior reduplicant (*DU*), the two are ulnopalmar images. Ventral views. Arrow points anteriorly. × 11.

Fig. 8 Eight days after operation, secondary limb growing out from base of primary, closely anterior to it.

Fig. 9 Twelve days after operation, secondary limb becoming fused with primary, former shows two digits; latter, three.

Fig. 10 Eighteen days after operation, stage 46. Secondary limb curves over from ulnar border of primary so that the palms face each other.

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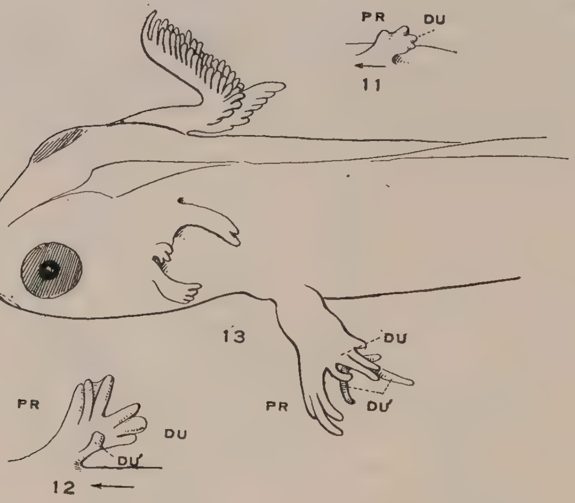
[10] Brandt ('24) finds that both the anteroposterior and dorsoventral axes in the limb of Triton taeniatus are determined by the time the embryo reaches the tail-bud stage. From the results of limb grafts in earlier stages, this author concludes "Die Triton-Neurula ist also dem Amblystoma-Schwanzknospenstadium biologisch äquivalent" (S. 549). Likewise Milojevic ('24) finds that all three axes are determined very early in regenerating limb buds of Triton cristatus. Gräper's ('22, '23, '24) experiments indicate that the stages at which determination of the various axes in the anuran hind limb takes place are somewhat more widely spaced.

point out at this time that radial reduplications are more common than ulnar (table 3), and that primary dorsal and palmar relationships between components are relatively rare. If both radial and ulnar reduplicants are present in connection with the same primary limb, the former almost invariably develops first. Furthermore, when a reduplicant develops relatively late, it is always on the ulnar side of the primary. In a study of the data concerning the development of 116 reduplicants, eighty-three were found to be radial and thirty-three ulnar. The average time after operation at which the radial reduplicant appeared was 5.38 days before



Figs. 11 to 13 Experiment Het. d. d. 16. Right limb rudiment to left side, dorsodorsal orientation. Three limbs resulted, primary (*PR*) reduplicated posteriorly, and this secondary limb (*DU*) later formed a tertiary member (*DU'*). × 11.

Fig. 11 Primary and secondary members mirrored in the radial plane. Arrow points anteriorly. Ventral view, eleven days after operation.

Fig. 12 Primary and secondary members each with three digits, tertiary bud appearing on posterior border of complex. Arrow points anteriorly. Ventral view, seventeen days after operation.

Fig. 13 Laterodorsal view of definitive complex. Primary and secondary limbs mirrored in the radiopalmar plane, tertiary limb mirrored ulnopalmar with secondary. Thirty-five days after operation.

the average time at which the ulnar become visible. If these facts are taken in conjunction with the well-known order of formation of the digits in the radio-ulnar direction,[11] the activity of the limb in the formation of buds, both normal and abnormal, is seen to progress from the radial to the ulnar border.

TABLE 3

Showing the mirror planes between the components of duplex and triplex limbs

OPERATION	TOTAL	PRIMARY LIMB			SECONDARY LIMB			MIRROR PLANE					LIMB REDUPLICATED		TERTIARY LIMB			MIRROR PLANE				
		Harmonic	Disharmonic	Not determined	Harmonic	Disharmonic	Not determined	Radial	Palmar	Ulnar	Dorsal	Not determined	Pr. or Du.	Total	Harmonic	Disharmonic	Not determined	Radial	Palmar	Ulnar	Dorsal	Not determined
Hom. d. v.	45[1]	4[2]	31	10	39	1[3]	5	28	0	3	1	13[4]	Pr.	4	4	0	0	0	1	3	0	0
													Du.	2	1	1	0	1	0	0	0	1
Het. d. d.	24[5]	0	16	8	16	0	8	17	0	7	0	5	Pr.	5	2	0	3[6]	0	0	3	0	2
													Du.	3	0	1	2[6]	0	0	1	0	2[6]
SE[8] (Hom. d. v.) (Het. d. d.)	9	0	8	1	9	0	0	7	0	0	0	2[6]	Pr.	1	1	0	0	0	0	1	0	0
													Du.	0	0	0	0	0	0	0	0	0
IE[8] (Hom. d. v.)	18	0	8	10	15	0	3	12	0	1[7]	1[7]	0	Pr.	7	6	0	1	0	2	4	0	1
													Du.	0	0	0	0	0	0	0	0	0

[1] Fifty-six cases of young double buds and thirty-one cases in which the secondary limb became single through resorption of the primary are omitted.

[2] By rotation.

[3] Image of a primary limb harmonic by rotation.

[4] Including three cases of two harmonic limbs in series.

[5] Exclusive of three cases of reduplication with subsequent resorption of the primary bud, also two preserved in young stages.

[6] Hyperdactyly.

[7] Due to faulty posture.

[8] These twenty-seven cases are taken from Professor Harrison's series of double limbs resulting from superposed rudiments (*SE*) and simple inverted buds (*IE*).

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[11] The normal digits are formed progressively from the radial to the ulnar border, digits 1 and 2 being the first distinguishable, 3 and 4 (and 5) appearing later in the order named. This seems to be generally true in the pentadactyl limb, as Strasser ('79) has shown in a number of urodeles and Sewertzoff ('07) in reptiles and urodeles. In the mammals the buds for the digits all appear simultaneously, but this may probably be considered a shortening of the ancestral process of progressive budding.

B. Anatomy of the double limb. Braus ('09) found that when double limbs resulted from a single transplanted fore-limb rudiment in Bombinator the girdle was also reduplicated to a greater or lesser extent. Detwiler ('18) has shown that in Amblystoma the rudiments for various parts of the girdle are localized and differentiated as a mosaic early in development. The central portion of the girdle rudiment is carried over in transplantation of the typical limb disc and develops as such in its new location. The outlying portions which are not removed develop in situ and by a process of hyperplasia may draw together into a single plate, thus forming a girdle which is topographically complete (Harrison, '18). According to Detwiler ('18, '20), double limbs resulting from heterotopic transplants do not possess double girdles, and a single limb formed from a transplant to normal position may form a nearly normal girdle from the contributions of both the host and the transplant. In case the resulting limb is disharmonic, its girdle may be disharmonic also.

The present study shows that in case a double limb results from the transplantation of a rudiment to normal location, the girdle likewise tends to be doubled. These duplicities are of various kinds, though the coracoid is the element most often involved. In no case were two entirely separate girdles derived from the same limb bud. The case shown in figure 14 is the nearest approach to this condition. Here a large, fairly well-formed left girdle (*G′*) is seen fused anteriorly with a qualitatively normal, though small, right girdle (*G*). The two glenoid cavities are entirely separate and are situated in a normal position. No definite procoracoid element can be identified in the posterior member and the most dorsal part of the suprascapula is missing. The primary limb (figs. 8 to 10, *PR*) articulates with the posterior glenoid cavity, the secondary with the anterior.

Figure 15 shows a closer union of girdles. The humerus articulating with the anterior glenoid cavity belongs to the primary limb, a left whose manus is reduplicated externally. There is also internal doubling in the forearm and the humerus is massive. Articulating with the posterior glenoid cavity is the normal harmonic limb developed from the reduplicating or secondary bud. We have here a peculiar condition in which a left limb articulates with a right girdle and a right limb with a girdle which is apparently a left.

An interesting massive girdle is that shown in figure 16. In this case the secondary limb rapidly forged ahead and the primary was resorbed almost completely. On sectioning, the latter was seen to be represented by an accumulation of procartilage cells (*HPR*). The girdle consists of a very wide coracoid and scapula, the former being rather foreshortened dorsoventrally and the latter surmounted by two small and abortive suprascapulae. There is no glenoid cavity for the anterior (resorbed) limb (*HPR*) and the posterior (*HDU*) is somewhat abnormally placed on the extreme caudal margin of the girdle. The form and relationships of this compound structure indicate that we have here a left (anterior) and a right (posterior) girdle fused together by their anterior margins. If this interpretation is correct, the condition is the opposite of that in the previous case.

Figure 17 shows an interesting girdle the anterior portion of which receives the anterior limb, a normal right,[12] at *G*. Posteriorly and ventrally from this region stretches an almost exact duplicate of the anterior coracoid and procoracoid. This structure bears a small cup-shaped depression for the pointed head of the posterior humerus. The shape and position of the two coracoidal elements strongly suggest that both girdles are rights. This would be in harmony with the limbs, as these are both rights also.

From these conditions of more nearly total duplicity of the girdle a graded series is found with the accessory girdles growing smaller and more closely fused with the normal. The glenoid cavities may fuse together into a single enlarged fossa

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[12] This particular case is not figured, but it differs from the one shown in figure 6 only in the presence of one less digit on the posterior limb. The girdle belonging to this limb is similar, though it shows less extra tissue and the secondary member is not articulated with it.

(fig. 18, *G*) to accommodate the immense head of a massive humerus (compare figs. 11 to 13), and in limbs in which doubling does not involve the proximal part of the humerus, the glenoid cavity is normal.

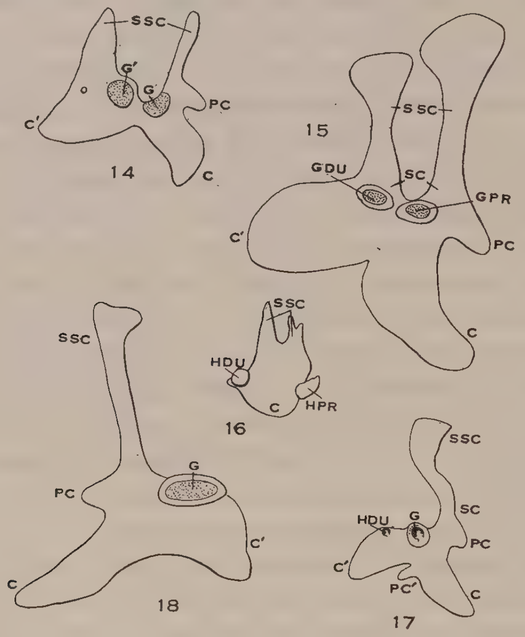

Fig. 14 Girdle from experiment Het. d. d. 34 (compare fig. 10) from a dissection after staining it in methylene blue. Anterior girdle (*G*) nearly normal, posterior (*G′*) a left. Eighteen days after operation. × 22.

Fig. 15 Girdle from experiment Het. d. d. 44 (limbs type C, fig. 7). From a dissection after staining in methylene blue. Anterior primary-tertiary complex articulates at *G PR*, secondary limb at *G DU*. Whether posterior girdle is right or left is uncertain. Thirty-six days after operation. × 22.

Fig. 16 Girdle from experiment Het. d. d. 49 from a graphic reconstruction from serial sections. *H DU*, humerus of secondary limb, poorly articulated. *H PR*, small degenerate humeral head of primary limb, no glenoid cavity. Thirteen days after operation. × 22.

Fig. 17 Girdle from experiment Hom. d. v. 54 (limbs not figured, but almost identical with Hom. d. v. 46, fig. 6). From a dissection after staining with methylene blue. The normal primary limb (harmonic by rotation) articulates at *G*. The abnormal secondary limb (a right) articulates abnormally at *H DU*. Twenty-six days after operation. × 22.

Fig. 18 Girdle of experiment Het. d. d. 16 (right limb to left side). Compare figure 13. From a dissection after staining in methylene blue. Solid double humerus articulates with enlarged glenoid cavity *G*. A supernumerary coracoid (*C′*) is present. Thirty-five days after operation. × 22.

In some of these double limbs, especially where development was slow and irregular, marked structural deficiencies in the girdle are manifest. In these cases it is probable that the rudiments for the missing parts were removed at operation and that the deficiency was not made good by the transplant. Inasmuch as the girdle rudiment is a mosaic, the formation of the doubled structures is somewhat unexpected. It is likewise remarkable that some of the cases should produce supernumerary structures which are so near topographical perfection.

The literature contains no account of the morphology and development of the skeleton of the free limb in Amblystoma, but from evidence gathered during the present study there is no material difference in this respect between this form and other urodeles. Strasser ('79), Goette ('79), Rabl ('01), and Sewertzoff ('04, '07) have given excellent accounts of the development in a variety of forms, and the figures of the carpus of Salamandra maculosa given by Gegenbaur ('64) resemble very closely the arrangement in Amblystoma. In the developing limb differentiation of the skeletal anlagen takes place in a proximodistal direction and from the preaxial to the postaxial border. This is likewise true in the regenerating limb (Goette). Strasser and Goette differ concerning certain details in the arrangement of the procartilage axis, but the accounts are in fundamental agreement.

There is every à priori reason why the skeletal axes of double limbs should develop along the same lines as the normal, and in general this has been found to be true. Each separate member has a normal cartilaginous axis. When doubling involves only a part of the limb, the skeletal reduplication is correspondingly diminished. The more closely fused reduplications may show a single median radius flanked by two ulnae (if the mirror plane is radial) or they may be so intimately united that only one central cartilage, presumably a compound ulna, is present.

In transplanted limbs where the girdle is normal or nearly so, the muscles are also, as a rule, practically normal (Detwiler, '20). Those attaching the free limb to the girdle and those extending between the free limb and the body wall grow proximally from the base of the blastema to their anatomical 'origin.' When these regions of attachment are lacking, the muscles spread out in fan-shaped fashion in the connective tissue. In such cases the function of the limb is naturally limited, even though the muscles may be present and normally innervated. Often, however, such muscles gain attachment to some part of the girdle with which they are not normally associated, and so are able to function in varying degree.

The normality of the musculature developed in connection with double limbs is correlated to a certain extent with the degree of duplicity of the skeleton (table 4). The dorsal muscles (m. dorsalis scapulae and m. latissimus dorsi) are usually single and very nearly normal, just as the scapula and suprascapula are more rarely duplicated than other parts of the girdle. When these skeletal parts are duplicated, the m. dorsalis scapulae is the more likely to be doubled.

Detailed studies have not been made of either the normal or the supernumerary intrinsic musculature, but in general it likewise corresponds to the arrangement of the skeletal parts. In the regions of convergence of fibers from the two or more limbs there is more or less intermingling, but attachments are very nearly normal in so far as there is room for them to be.

The innervation of the limb is dependent on the position and extent of the rudiment at the time when the nerves begin to grow out (Harrison, '07). In Amblystoma this corresponds to the third, fourth, and fifth spinal nerves. Detwiler ('20, '22) has shown, however, that if limbs are transplanted posterior or anterior to their normal position, there is a tendency for them to be supplied by nerves from the normal region of the cord. The more posterior or anterior nerves may also become involved if the transplant is sufficiently remote from its normal location.

When reduplications are produced from a single limb rudiment in normal location, it is obvious that the resulting limbs

TABLE 4

Showing relationship between muscle and girdle duplicity in double limbs. Examples chosen at random

OPERATION	GIRDLE				MUSCLES						
	Supra-scapula	Scapula	Coracoid	Pro-coracoid	Dorsalis scapulae	Latiss. dorsi	Supra-cor-acoideus	Pro-coraco-humer-alis	Pectoralis	Sub-coraco-scapu-laris	Coracobra-chial. long. and brevis; ancon.
Het. d. d. 10	Normal	Normal	Small double	Small	Normal	Normal	Short double	Short	Normal	Normal	Normal
Het. d. d. 11	Normal	Normal	Small double	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Het. d. d. 16	Normal	Normal	Double	Normal	Normal	Normal	Double	Normal	Normal	Normal	Normal
Het. d. d. 23	Normal	Normal	Absent	Absent	Normal	Normal	Present	Present	Present	Normal	Normal
Het. d. d. 34	Double	Double	Double	Single	Single	?	Double	Present	Single	Large	Normal
Het. d. d. 36	Nearly normal	Displaced caudad	Small isolated	Small isolated	Present	Present	Present	Present	Present	?	Normal
Het. d. d. 43	Normal	Normal	Double	Small	Single	Single	Double	Small	Single	?	Double
Het. d. d. 44	Double	Double	Double	Single	Double	Single	Double	Normal	Double	?	Double
Het. d. d. 48	Small isolated	Absent	Small isolated	Absent	Abnormal	?	Small	Abnor-mal	Abnormal	?	?
Het. d. d. 49	Double	Compound	Compound	Absent	Double	Single	Double	?	Single	?	Present
Hom. d. v. 5	Isolated	Present	Large	Present	Short	?	Single	Single	Single	?	?
Hom. d. v. 8	Single	Single	Double	Single	Normal	?	Double	Present	Present	Normal	Normal
Hom. d. v. 15	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hom. d. v. 46	Normal	Normal	Double	Single	Normal	Abnormal	Double	Present	Present	Single	Single
Hom. d. v. 50	Normal	Normal	Double	Single	Normal	Normal	Double	Normal	Normal	?	?
Hom. d. v. 54	Normal	Normal	Double	Double	Normal	Normal	Double	Normal	Normal	Normal	Normal
Hom. d. v. 139	Normal	Large	Normal	Normal	Double	Abnormal	Double	Normal	Normal	?	?

would never be very far apart, and one could not expect to find very marked variations from the normal segmental nerve contribution to the brachial plexus. All the double limbs examined in this study are innervated by a normal brachial plexus if their components are fused at all. When the members of the reduplication are completely and rather widely separated, one or the other (sometimes both) is displaced somewhat from the normal location. In such cases the second nerve, in addition to the normal third, fourth, and fifth, may enter into the formation of the brachial plexus. Though anastomoses between all four of these nerves are effected, the bulk of the innervation of the more anterior limb is provided by the second and third, while the fourth and fifth supply mainly the posterior member. In so far as possible, the branches from the plexus are normal, but in the event of muscle duplicity, branches of the nerves are split off to supply the extra muscle bundle. In no case were the components of a reduplicated limb far enough apart to secure a more extensive involvement of the segmental nerves.

II. Experiments in splitting the limb bud

In the course of his experiments on the fore limb of Amblystoma, Harrison ('18) attempted to produce double limbs by a deep median incision through the limb disc and the removal of a narrow strip of ectoderm and mesoderm from this region. In no case was a double appendage produced by this method, although Tornier ('05), using older anuran larvae in which the separated halves of the rudiment could less easily grow together, had obtained many multiple appendages. Experiments of this kind were repeated with results identical with those obtained by Harrison. It was found, however, that by transplanting a piece of indifferent tissue of suitable size into the wound, the limb area was divided so that in a certain number of cases two limbs were formed, one from either side of the split.[13] In some cases a narrow strip

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[13] Detwiler ('22) figures two limbs of the same asymmetry, both of which were derived from a single rudiment transplanted to the gill region. The more

through the middle of the disc was removed, in others a slit was made in the desired direction through the limb area, the edges of the wound drawn apart, and the transplant healed in place. The transplants used consisted of long narrow strips of ectoderm with the subjacent mesoderm from the flank of an animal of the same age as the host, which had been lightly stained intravitam with Nile-blue sulphate. In some of the experiments the barrier also included a segment of the pronephric duct and some myotome tissue. A stained transplant was used to facilitate accurate observations on its placement and on the location of the limb buds relative to the split after complete healing had occurred.

A considerable number of experiments were made splitting the limb buds in various directions, using stage 27-34, in which the limb bud is not visible externally, and stage 38-39, in which it is visible. In the first group the limb area was split in a, 1) dorsoventral, 2) anteroposterior, 3) anterodorsal-posteroventral, or a, 4) posterodorsal-anteroventral direction (fig. 19). In each case an attempt was made to divide the limb-forming cells as nearly equally as possible. The results are shown in table 5. In the second group of experiments with embryos of a more advanced age, the bud was divided in two different directions only (fig. 20).

C. Rudiments split in stages 27-34. Within these limits the asymmetry of the limbs which develop from a split rudiment does not vary with the age of the embryo[14] or the direction of the split.

The extensor surface of the definitive limb is derived from a more dorsal part of the embryonic limb disc than the flexor

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posterior has a well-developed girdle independent of the fragmentary suprascapula and coracoid formed in normal location. The anterior is associated with a rudimentary coracoid connected with the posterior girdle by a cartilaginous structure interpreted as a modified gill bar. A well-formed branchial bar which is present between the bases of the two limbs Detwiler considers may have split the rudiment. The second, third, and fourth nerves unite in the brachial plexus, the former mainly supplying the anterior limb, the two latter the posterior. No reduplicant is present on either limb.

[14] The few cases split in stage 36 indicate that the limb is considerably further differentiated at this time. Further experiments are necessary on this and on closely related stages.

surface (Swett, '23). Hence, if a split (anteroposterior) separating these halves of the rudiment resulted in the formation of two primary[15] limbs of opposite asymmetry,[16] it seems logical that these limbs might be expected to exhibit dorsal or palmar mirror relations, depending on which of the two became reversed. Similarly, a dorsoventral split and subsequent reversal of asymmetry in one primary member should

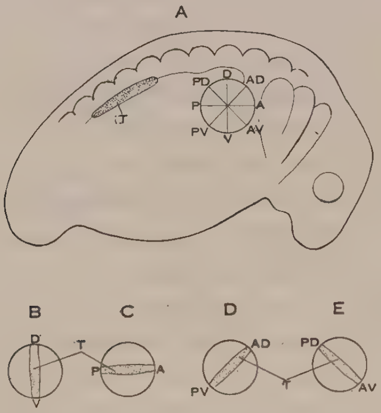

Fig. 19 Diagrams to show the direction of splitting the limb rudiment and the method of inserting a transplant to prevent fusion of the parts separated by the operation.

A. Outline of Amblystoma embryo in stage 29. The circular area represents the limb rudiment; the letters on its circumference, the points used in the series, and the lines bisecting it, the direction of the split in the four series. The outline of the transplant is shown at *T*. Abbreviations: *D*, dorsal; *V*, ventral; *A*, anterior; *P*, posterior; *AD*, anterodorsal; *PD*, posterodorsal; *PV*, posteroventral; *AV*, anteroventral; *T*, transplant.

B. Transplant in place after a dorsoventral split.

C. Transplant in place after an anteroposterior split.

D. Transplant in place after an anterodorsal-posteroventral split.

E. Transplant in place after a posterodorsal-anteroventral split.

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[15] Both limbs developing, one from each half of the split rudiment, are designated as 'primary,' although they do not always appear at the same time or develop with equal rapidity. Thus, when the limb rudiment is split in a dorsoventral direction, we have an anterior primary and a posterior primary member; if the division has been made in an anteroposterior direction, we have anlagen for a dorsal and a ventral primary limb.

[16] Compare Tornier ('05), who reports reversal of asymmetry in one of the limbs resulting from a split bud in Bombinator.

result in a pair of radial or ulnar images, since the cells destined to form radial tissue seem (Harrison, '21; Swett, '23) to be placed anterior to those destined for the ulnar side of the limb. The 'expected' limbs and those which actually develop are shown diagrammatically in figure 21. There is no reversal of asymmetry in either of the two primary limbs. Both are of the same side of the body and not mirror images of each other. In each case the more posterior[17] primary limb develops a reduplicant of opposite asymmetry on its radial border—the side facing the other primary member. The definitive complex thus conforms to Bateson's rules and corresponds to Przibram's Bruch-dreifachbildungen.

TABLE 5

Summary of experiments in dividing the limb rudiment in different directions

OPERATION	TOTAL	POSITIVE	RUDIMENT NOT PERMANENTLY DIVIDED			RUDIMENT PERMANENTLY DIVIDED							
						One bud resorbed		Both buds developed					
								Anterior primary			Posterior primary		
			Limb blocked	Single limb normal	Reduplicated	Other single	Other reduplicated	Total	Harmonic	Reduplicated	Total	Harmonic	Reduplicated
A$_{27-34}$ d/v	59	39	8	10	2^1	4	8	7^1	7	0	7	7	7
								Ventral primary			Dorsal primary		
A$_{27-34}$ a/p	79	72	3	36	7	9	10	6^1	6	0	6	6^2	3
								Ant.-ventr. primary			Post.-dor. primary		
A$_{34}$ ad/pv	10	10	0	5	1	1	3	0	0	0	0	0	0
								Post.-ventr. primary			Ant.-dor. primary		
A$_{34}$ pd/av	10	8	1	3	0	0	2	2	2	1^3	2	2	1

[1] Including one case of hyperdactyly.
[2] Including three spur-like limbs.
[3] Experiment A34 pd/av 5, see text, page 450.

Transverse serial sections made at the time the developing buds first appear as definite swellings on the side of the body show a condition such as that seen in figure 22. In the case figured the ventral bud is somewhat further developed than the dorsal. Between the buds a fragment of the transplanted pronephric duct forms a definite barrier.

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[17] One case which shows the reduplicant on the ventral limb is not entirely an exception as would seem at first (p. 450).

Immediately on its appearance each of the primary limbs assumes a posture as nearly normal as possible, considering its position on the body and the proximity of its twin. The two limbs usually remain separate throughout development. Before digital bifurcation occurs in either primary limb, a supernumerary bud becomes visible on the anteroventral border of the posterior member. This reduplicant developed in every case in the present series and invariably formed a disharmonic limb. The complex thus formed subsequently becomes closely fused, union of the two components usually taking place through the radial digit. This extra limb often develops when one of the primary limbs has been resorbed.

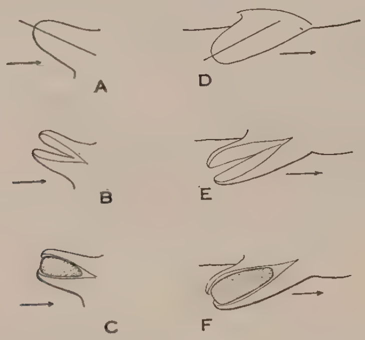

Fig. 20 Diagrams to show the direction and method of splitting the limb bud in older stages (stages 38–39). The lateral views A, B, and C represent three steps in the placing of the transplant (stippled) in a split perpendicular to the sagittal plane of the larva and oblique to the frontal (series Ax r/u). The dorsal views D, E, and F represent similar stages in the process of splitting the bud in a plane perpendicular to the frontal and oblique to the sagittal (series Ax e/f). The arrows point toward the anterior end of the larva.

In many instances only one primary limb developed after an attempt had been made to split the rudiment. This indicates that the potency of one bud must not exceed that of the other beyond a certain small amount if the two systems are to be sufficiently balanced to produce complete limbs.

1. Rudiments split dorsoventrally. A series of fifty-nine animals was kept under observation for the appearance of limbs from a rudiment which had been divided in a dorsoventral direction by the method described above. As may be seen from table 5, all limb growth was entirely prevented[18] in eight specimens in this series. The derivation of the single limbs which form in this group depends on the direction of displacement of the transplant to cover the anterior or posterior portion of the rudiment. When development of the posterior portion is blocked, the limb produced from the anterior part of the disc is invariably small and considerably displaced ventrally at first. Dissection of the girdles of such limbs shows them to be proportionate in size to the limb.

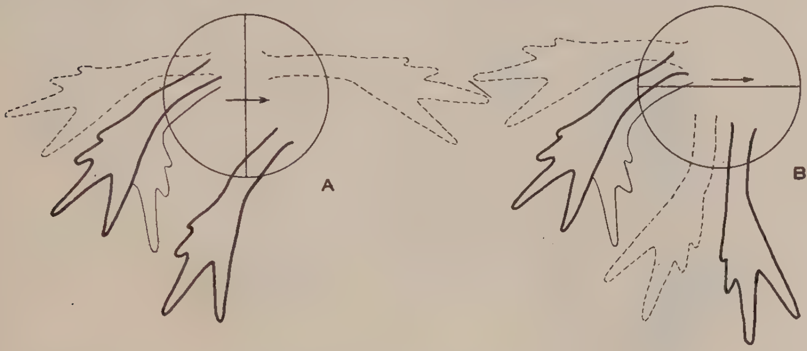

Fig. 21 Diagrams to show the results of the two operations, splitting the limb rudiment dorsoventrally (A), and anteroposteriorly (B), represented as on the right side of the embryo. The arrows point to the anterior end of the animal. The circles represent the limb rudiments, the line through the center the direction of split. The limbs which develop are shown in profile, a heavy outline indicating the primary members, a light outline the reduplicating ones. The broken outlines show the limbs which would have developed had one of the primary members suffered reversal of asymmetry.

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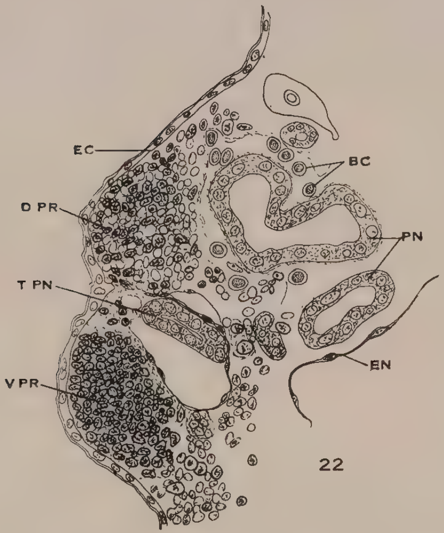
[18] Harrison ('18) found that regeneration of the limb after removal of the rudiment could be blocked by covering the wound with indifferent tissue, but in his experiments the limb area was carefully cleaned of mesoderm cells. The conditions in the present series are quite different, as all or nearly all of the limb mesoderm is left intact and merely divided into approximately equal parts. In the only case studied histologically, no trace of either limb or girdle tissue could be found in serial transverse sections.

Qualitatively, both are entirely normal. Limbs of this type are less common than the practically normal limbs formed from the posterior part of the disc when the transplant has slipped anteriorly in healing. A few cases appear in which a reduplicated limb formed as a result of a disturbance other than the splitting of the rudiment. The limb resulting in the case shown in figure 23 is a reduplicated structure composed of a massive base and two separate hands mirrored in the ulnar plane. The primary limb is a right and the secondary, a left. What induced the formation of the secondary limb in this case is not known, but it is certain that it did not develop from any part of the original rudiment isolated by the transplant.



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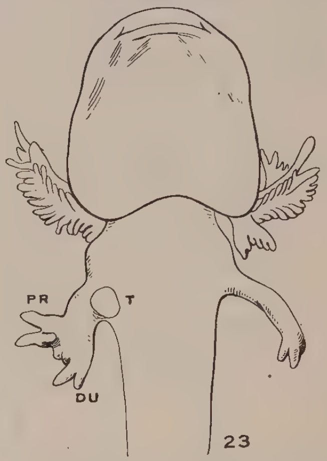

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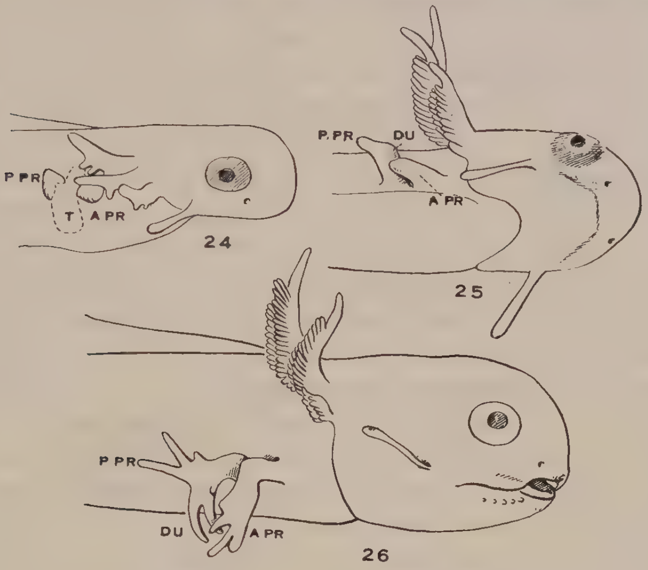
Fig. 22 Experiment A34 pd/av 6. Transverse section through dorsal and ventral primary buds derived from splitting a limb rudiment in stage 34. Three days after operation (stage 39). × 50. *EC,* ectoderm; *EN,* endoderm; *BC,* blood cells; *PN,* pronephros; *TPN,* section of pronephric duct used to split the rudiment; *D PR, V PR,* dorsal and ventral primary limb buds.

Fig. 23 Experiment A29 d/v 14. Limb reduplicated symmetrically as a result of general operative disturbance. The transplant (*T*) used in an attempt to split the rudiment did not heal in properly and remains at the base of the limb. The anterior limb is primary (*PR*) and harmonic, the reduplicant (*DU*) stands as its ulnar image. Nineteen days after operation. × 11.

We now come to the cases in which two limbs are produced, one from either side of the split. As a rule, the anterior is smaller than the normal and in some cases is the less advantageously placed of the two on the body of the animal. On the other hand, this limb articulates with the normal girdle, while the posterior, though often no less normally oriented, articulates with the girdle very abnormally, if at all.



Figs. 24 to 26 Experiment A29 d/v 34. Right limb rudiment split dorsoventrally in stage 29. Two primary limbs (*A PR* and *P PR*) resulted, and the posterior reduplicated on its radial border. The first digit of this symmetrical complex is represented by a small nodule on the medial surface, not visible in the drawings. × 11.

Fig. 24 Five days after operation. Both primary buds are plainly seen with the outline of the transplant between them.

Fig. 25 Nine days after operation. Both primary limbs are developing rapidly, the posterior shows a reduplicant (*DU*) on the anterior border.

Fig. 26 Twenty-three days after operation. Both the single anterior primary limb and the posterior complex are well developed. The anterior limb is the functional member.

Figure 24 shows the limbs as they appear five days after the operation in a case typical for this series. The animal is at approximately stage 40 and both limbs are plainly visible with the transplant between them. The anterior (*A PR*) is the smaller, a relationship which it held throughout development, and points somewhat more directly posteriorly than normal. The posterior bud (*P PR*) is practically normal in size and appearance, although situated somewhat posterior to normal and pointing rather abruptly dorsally. The same relative position was retained, and four days later (fig. 25), the tip of the posterior limb has turned posteriorly and the prominence (*DU*) on its anterolateral border forecasts the appearance of a radial reduplicant. After four more days, both the primary limbs were seen to be definitely rights (harmonic). The two members of the posterior complex were mirrored in the radiodorsal plane. Beginnings of three digits were seen on the anterior limb, while each component of the posterior complex bore two. Twenty-three days after the operation, the animal was preserved. The limbs are shown in figure 26. The anterior primary limb is the functional appendage and practically normal in all respects. The posterior complex is symmetrical and composed of seven digits. The middle one of these is not visible in the figure. The girdle elements which bear these limbs are described on page 453.

Figure 27 shows an animal in which the portion of the disc anterior to the split failed to form a limb. A definite nodule representing the anterior primary member (*A PR*) was observed anterior to the transplant on the seventh day after the operation, and it remained without increase in size until the animal was preserved one week later. The posterior bud developed normally for about nine days, and then a reduplicant appeared on its anterior border. The two components are mirrored in the radiodorsal plane and constitute a symmetrical appendage exactly comparable to the posterior complex formed when the anterior primary limb actually developed.

2. Rudiments split anteroposteriorly. This series consists of seventy-nine experiments in which attempts were made to divide the limb rudiment anteroposteriorly. Accurate division is more difficult to accomplish in this operation, presum-

ably because the majority of the limb-forming cells are located in the dorsal half of the disc and because of the absence of definite landmarks for guiding the incision in this direction. In more than half of the cases a perfectly normal single limb was produced. Of the three limbless animals in this series, two developed small buds which were later resorbed. In the group of reduplicated limbs recorded as seven in table 5, the reduplicant possessed digits in only one case; in the others it is spur-like and its asymmetry cannot be determined.

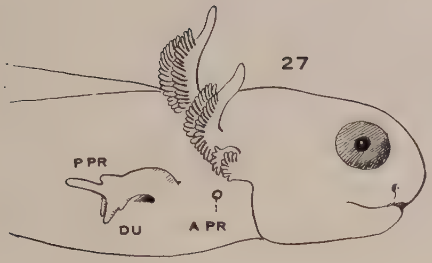
In the six cases showing development of both primary buds the dorsal bud formed an appendage with normal digits in only two instances. In both of these the limb became reduplicated on the anteroventral border exactly as in the dorsoventral series. In every case the two primary limbs were of like asymmetry, but the percentage of normal appendages formed in this series is much smaller than in the preceding one. Five of the six ventral primary limbs which developed in the absence of the dorsal are normal, with the exception of one case of hyperdactyly. The other, though abnormal, is definitely harmonic. Five of the ten cases where reduplication of the remaining limb occurred after resorption of one bud, show small or irregular supernumerary parts. The others show typical radiodorsal (or radiopalmar) mirror relations.

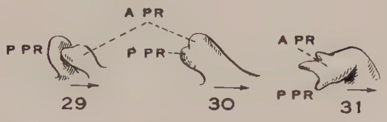
The limbs formed in this series are similar to those in the dorsoventral, differing only in the relative positions of the primary limbs (fig. 28) and in the larger number of abnormalities produced. The findings indicate that injuries to the dorsal half of the rudiment more often result in abnormalities than do injuries to any of its other parts. Neither limb is able to assume a position as nearly normal as is sometimes secondarily gained by the limbs in the dorsoventral series, and in consequence the anatomical relations are less perfect and the function proportionately impaired.

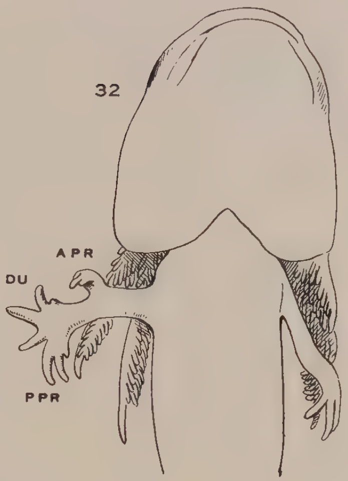
3. Rudiments split obliquely. The experiments in which the rudiments were split obliquely compose two series of ten

animals each (table 5). Eighteen of the twenty cases are positive; in ten of these the attempt to split was unsuccessful, though in one case the one primary limb which formed became reduplicated as a result of the operation. In one case the limb was blocked. In eight cases two primary limbs appeared and, as the table shows, their behavior was in all respects, save one, exactly like that of the primary limbs in the dorsoventral and the anteroposterior series. This exception is the one case in which a reduplicant was developed on the ventral primary limb. This limb did not appear until stage 43, nearly two weeks after the other, but it developed into a right and a small reduplicant formed near the elbow on the ulnar border. It is to be noted that the extra member was between the two primary limbs and undoubtedly would have developed into a disharmonic member had the animal not died. Furthermore, it is of interest that the dorsal (normal) limb in this case articulated with a nearly normal though small girdle. The ventral limb had no connection with the girdle. These facts were made out by dissection after staining the animal with methylene blue. This case gives additional evidence that the more nearly normal a limb is, the less liable it is to reduplication.

A peculiar feature of the cases of resorption observed in these series is that a large percentage of them occurred in the limb which would have been the functional appendage and very nearly normal if it had developed. In none of these instances was the default of the potentially functional limb compensated for by the other primary limb—a result hardly to be expected in view of the poor articulation of the latter with the girdle and its not infrequent possession of a reduplicant. The whole process is decidedly adverse to any teleological explanation. The reduction seen in the more anterior limbs was possibly brought about by reason of their disadvantageous situation during early development. On the other hand, the relative positions of the two primary limbs were sometimes interchanged during development, the one which started out in an abnormal position later becoming more normal and vice versa.







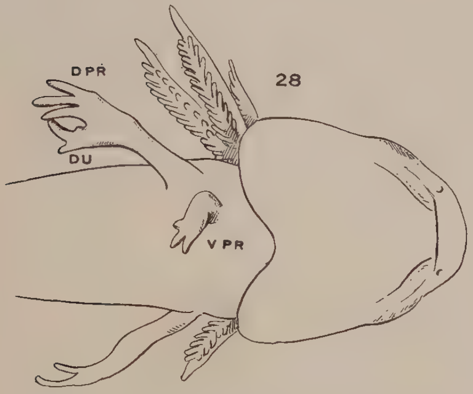


Fig. 27 Experiment A34 d/v 42. Right limb rudiment split dorsoventrally in stage 34. Fourteen days after operation. Two primary limbs started to grow, the anterior (*A PR*) was reduced to a small nodule, the posterior (*P PR*) reduplicated symmetrically. × 11. The digit shown in the background is the multiple first digit common to both members of the complex.

Fig. 28 Experiment A34 a/p 62. Right limb rudiment split anteroposteriorly in stage 34. Two primary limbs developed, the ventral one (*V PR*) is small and placed rather far down on the ventral surface of the body. The dorsal one (*D PR*) is well developed and reduplicated symmetrically on the radial border. The first digit of the reduplicant is reduced in size. Twenty-five days after operation. × 11.

Figs. 29 to 31 Experiment A30 d/v 13. Right limb rudiment split dorsoventrally in stage 30. Two primary limbs resulted, but soon fused to form a short, thick, compound limb. × 11.

Fig. 29 Five days after operation.

Fig. 30 Nine days after operation, close fusion.

Fig. 31 Nineteen days after operation. Posterior primary limb forms radial digit (*P PR*) and part of compound proximal parts of the limb.

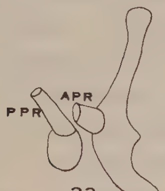
Fig. 32 Experiment A29 d/v 31. Right limb rudiment split dorsoventrally in stage 29. Two primary limbs developed, the posterior (*P PR*) reduplicated anteriorly, forming a symmetrical manus, the anterior (*A PR*) grew up close to the base of the posterior and fused with it. The skeletal parts of the two are separate. Twenty-eight days after operation. × 11.

Fusion of the two halves of the divided bud is, as we have seen, of practically universal occurrence unless a barrier is inserted. This is proved by a large number of cases in each group of experiments which are not included in the tabulations. In all such cases the two halves of the rudiment coalesce within a day or two with subsequent production of a normal limb. This type of early fusion we may designate as 'primary.' Another and somewhat rare type of fusion which occurs a little later in development may be designated as 'secondary' fusion. Ordinarily, the coalescence of the two buds is not quite so complete in this type and a somewhat massive compound limb may develop. Figure 29 shows the early appearance of two limbs, both of which are harmonic and rather close together. With later growth they became still more closely associated (fig. 30) into the production of a very thick compound limb (fig. 31). The posterior limb (*P PR*) assumes the position of a digit-like projection on the radial side of the anterior (*A PR*). Sections show the girdle to be single and practically normal.

On casual examination of the case shown in figure 32, the small harmonic limb (*A PR*) might be taken as derived from a bud from the anterior border of the posterior complex. This is in reality an example of fusion by inclusion, as this anterior limb appeared independently at the base of the posterior nine days after operation. It soon fused with the side of the larger limb at its point of attachment to the body, and the two became enclosed in the same skin sheath. With subsequent elongation of both limbs, the smaller came to occupy its definitive position as seen in the figure. The humerus of the anterior limb articulates with a single normal girdle and the compound humerus of the posterior complex has no complete articulation.

4. The limb girdle. The girdles formed in the split-bud series present a number of interesting details which differ in some respects from the conditions seen in connection with the spontaneously reduplicated type of double limb (p. 436). In general it may be said that the single primary limb is

borne on a girdle, which, if not normal, more nearly approaches normality than the one with which the reduplicated primary limb articulates. The animal shown in figure 26 presents a typical arrangement of the cartilages of the girdle. The anterior part of this girdle (fig. 33) is normal except for a reduction in the anteroposterior diameter of the coracoid, and the anterior primary limb articulates with a normal glenoid cavity. The posterior complex articulates with a glenoid fossa borne on a small plate of cartilage, representing chiefly the coracoid. There is no connection between the two girdles. The musculature of the girdle bearing the anterior limb is normal except for the m. pectoralis. This muscle has a normal origin and insertion, but its fibers are blended somewhat with a similar muscle bundle connected with the posterior complex. With the exception of this muscle and an abbreviated m. supracoracoideus whose fibers converge from the entire lateral surface of the small coracoidal plate to be inserted on the humerus, the girdle musculature of the pos-



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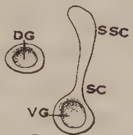

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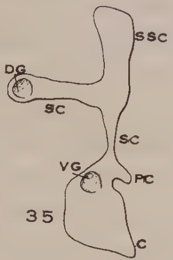

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Figs. 33 to 35 Girdles of limbs from split buds. From dissection after staining in methylene blue. × 22.

Fig. 33 Girdle from experiment A29 d/v 34 (compare fig. 26). Typical case. Anterior girdle normal, though reduced as to coracoid. Posterior a small coracoidal plate. Both humeri articulate normally. Twenty-two days after operation.

Fig. 34 Girdle from experiment A34 a/p 61. Fifteen days after operation. Ventral limb digitated but abnormal, articulates with *VG*, dorsal limb a spur articulates with *DG*, the latter borne on a plate scarcely larger than the head of the humerus. Coracoid and procoracoid lacking.

Fig. 35 Girdle from experiment A34 a/p 62 (compare fig. 28). Scapula rudiment divided, part separated at right angles to the other and bears glenoid cavity (*DG*) for the dorsal complex at its tip. Anterior girdle normal except for extreme thinning of scapula. Twenty-five days after operation.

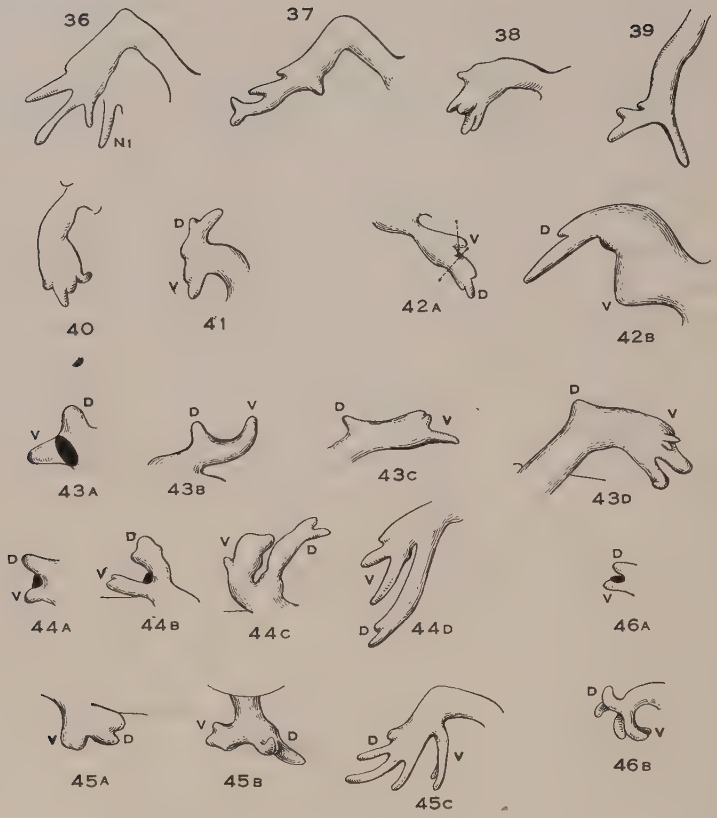
terior limb is wanting. From this typical condition various degrees of further malformation are seen. In several cases the head of the posterior humerus does not articulate with the girdle at all, but is held in place against the musculature of the body wall only by skin and connective tissue. Abnormalities of the anterior girdle also occur. These consist of absence of parts (fig. 34) or in a partial splitting (fig. 35). It is interesting to note that when the rudiment is split dorsoventrally, the coracoid (fig. 33) is the portion of the girdle most profoundly affected; when the direction of split is anteroposterior, the scapula (fig. 35) is the part involved. This falls in line with Detwiler's ('18, p. 503) account of the arrangement of the girdle rudiments in the tail-bud stage. The dorsoventral split as carried out in the present experiments passes through the center of the coracoid rudiment, while the anteroposterior split approximately bisects the scapula rudiment. Sometimes this injury causes division of the part, sometimes its suppression. When it is divided, each portion is smaller than normal, thus further corroborating Detwiler's findings that the girdle is a mosaic and not an equipotent system. The hyperplasia of the girdle so often noted (p. 434 and figs. 14 to 18) when the limb bud doubled spontaneously is not observed in any case in which the complex was derived by splitting the limb rudiment, although perhaps it might have occurred had the animals been allowed to live for a much longer period. In cases of resorption of the free appendage the girdle is usually formed in situ and often is nearly normal except that the glenoid cavity is not formed unless the humeral head is present.

Exactly as in the case of the spontaneously reduplicated limbs, the musculature which develops in connection with the double limbs derived from split rudiments varies directly with the degree of development of the limbs and of the girdles. In cases of reduction of one of the primary limbs to a spur, the extrinsic as well as the intrinsic musculature is practically lacking. In some cases a few tangled fibers pass from the base of such an abortive limb to mingle with the musculature of the body wall, but these fibers are always very thin, their proximal attachments unstable, and consequently function is lacking in the limb. Similar conditions are seen in those limbs whose distal parts are well formed, but which do not articulate with the girdle. In these cases, however, the extrinsic musculature is somewhat better developed. When only certain portions of the girdle are formed, there is a tendency for the muscles belonging to the regions represented to develop more normally than those of other regions. This is seen particularly in the dorsoventral group in which a portion of coracoidal tissue is often associated with the posterior complex. In such cases the m. supracoracoideus and m. pectoralis are more regularly present than any of the other muscles running to the shoulder-girdle. Abnormalities in the attachments of certain muscles are seen in the cases of the anteroposteriorly split series in which the dorsal complex is connected with a portion of the scapular rudiment (fig. 35). This brings the only skeletal support of the limb to a position directly anterior to the base of the free appendage, and in one case mm. dorsalis scapulae, procoracohumeralis, and supracoracoideus have grown directly forward and become attached to this cartilage bar and to adjacent portions of the anterior girdle.

There is no evidence whatever that the type of split has anything to do with the number or the designation of the muscles formed except indirectly through their relationship with the girdle.

Innervation of these limbs is complete and the component nerves and their relationships to one another differ in no respect from those supplying the double limbs described in the first section (p. 438). In animals which had undergone reduction of one of the primary limbs to the extent that only a small mass of undifferentiated cells marks its former situation, abundant nerve branches may be seen ending in a tangle near the base of this nodule.

D. Limb buds split in stages 38-39. A few experiments were made using somewhat older animals than those described



Figs. 36 to 46 Some of the types of abnormalities produced by splitting limb buds in stages 38 to 39 (compare fig. 20). All operations on the right side. × 11.

Fig. 36 Experiment A38 e/f 11 (split according to D, E, F, fig. 20). Note shortening of radial digit. Insert N_1 is normal left radial digit drawn to scale for comparison.

Fig. 37 Experiment A38 e/f 10 (split according to D, E, F, fig. 20). Radial digit short, misplaced, second digit bifid.

Fig. 38 Experiment A38 r/u 18 (split according to A, B, C, fig. 20). Lateral view, fourteen days after operation. Double hyperdactyly of second digit.

Fig. 39 Experiment A38 r/u 7 (split according to A, B, C, fig. 20). Dorsal view, twenty-four days after operation. Radial digit separated from others.

Fig. 40 Experiment A38 r/u 17 (split according to A, B, C, fig. 20). Ventrolateral view, fourteen days after operation. Hyperdactyly of radial digit.

Fig. 41 Experiment A38 r/u 23 (split according to A, B, C, fig. 20). Ventrolateral view, seventeen days after operation. Aberrant double manus, very irregular, interpretation uncertain.

above, because in these, however well isolated the parts of the tip of a bud may be after a split, the base must still be a common possession of the two. In case an influence is operative in the limb whereby one component may be induced to reverse its asymmetry, this common base gives ample opportunity for interaction between the two buds.

Fig. 42 Experiment A38 r/u 22 (split according to A, B, C, fig. 20). Very irregular formations, asymmetry uncertain.

Fig. 42 a Ventral view, seventeen days after operation. Both ventral and dorsal limbs amputated along lines designated.

Fig. 42 b Lateral view, fifty days after operation. *D* and *V* designate the regenerates from the corresponding parts amputated thirty-three days previously. Too irregular for interpretation.

Fig. 43 Experiment A38 r/u 8 (split according to A, B, C, fig. 20). Dorsal and ventral buds formed, grew irregularly, and dorsal was later resorbed.

Fig. 43 a Six days after operation, lateral view. Solid black area is the transplant.

Fig. 43 b Nine days after operation, dorsal view.

Fig. 43 c Fourteen days after operation, dorsal view. Dorsal bud is being resorbed, ventral growing irregularly.

Fig. 43 d Twenty-nine days after operation, dorsal view. Ventral limb a right, palmar surface directed dorsally. Dorsal bud a knob at elbow.

Fig. 44 Experiment A38 r/u 4 (split according to A, B, C, fig. 20). Two limbs resulted from split, the ventral is harmonic and the dorsal disharmonic, though irregular. Radial mirror relations.

Fig. 44 a Lateral view, three days after operation. Solid black area represents the transplant.

Fig. 44 b Ventral view, six days after operation. Ventral limb looks like a harmonic member, dorsal like a disharmonic.

Fig. 44 c Ventral view, fourteen days after operation. Both limbs are irregular.

Fig. 44 d Dorsal view, twenty-four days after operation. Ventral limb definitely harmonic, from position of elbow and arrangement of the two digits, the dorsal member is disharmonic in radial relations with the ventral.

Fig. 45 Experiment A38 r/u 21 (split according to A, B, C, fig. 20). Two harmonic limbs developed, the ventral one became reduced.

Fig. 45 a Dorsal view, nine days after operation, ventral limb has no digits.

Fig. 45 b Dorsal view, thirteen days after operation, both limbs with two digits and nodule for third. Both limbs are rights.

Fig. 45 c Lateral view, forty-three days after operation. Marked reduction of the ventral member, it has two digits and elbow well marked.

Fig. 46 Experiment A38 r/u 2 (split according to A, B, C, fig. 20).

Fig. 46 a Lateral view, three days after operation. The blackened area represents the transplant.

Fig. 46 b Lateral view, eighteen days after operation. Each limb with two digits, palms facing each other.

The radio-ulnar series (fig. 20, A, B, C) includes twenty-three animals; the extensor-flexor series (fig. 20, D, E, F), thirteen. A similar expectation of the relationship between the direction of the split and the mirror plane of the resulting compound limb was entertained as in the case of the limb rudiments split earlier, but here also no such relationship seems to exist.[19]

The most marked characteristic in this group of experiments is the tendency toward reduction of one of the buds. If the operation fails to halve the bud exactly, the smaller portion often becomes subordinated to the larger.[20] Again, if the healing of the transplant is faulty, it is sloughed off and a normal limb produced. Perfect limbs from these split buds rarely occur. Figures 36 to 46 show a group of these abnormal limbs. In a few cases the limb is actually doubled. The radial digit is doubled or reduced in nineteen cases out of the thirty-six. In the extensor-flexor series the radial abnormalities consist largely of a shortening of the first digit with occasional hyperdactyly. In the radio-ulnar series the most common variation from the normal is radial hyperdactyly. Apparently the tip of the bud at this stage is in large part destined to form the first digit. The development of the most nearly normal case of double limbs in these series is shown in figure 46. The two more lateral digits are larger in each case, and the ventral one of these is reduplicated secondarily. Judging from the forced posture of the limb and the position of the elbow, the complex is composed of two hands mirrored in the palmar plane with the more ventral one the harmonic member. Another case in which the components are more widely separated than in the foregoing is shown in figure 44, a–d. It will be seen that development of each limb was very irregular. The complex consists of two limbs mirrored in the radial plane, the posterior a right from the ventral (palmar) bud and the anterior a left from the dorsal or extensor bud. The formation of a massive and abnormally postured limb is seen in figure 43, a–d. This limb was derived from the palmar bud, the other appears at the elbow as a rounded spur. The palmar surface of the ventral member is turned dorsad and bears two small supernumerary digits. A case which is regular so far as the morphology of its parts is concerned, but decidedly irregular as regards relations, is shown in figure 45, a–c. The limb derived from the dorsal[21] part of the original bud developed digits first and is definitely a right. Digits were later in appearing on the ventral limb, but their arrangement as seen in figure 45 b shows this limb to be also a right. Reduction of this latter member began at once and only two digits formed. The final condition is seen in figure 45 c.

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[19] In a recent paper by Doctor Harrison, which is now in press but which he permitted me to read in manuscript, considerable doubt is cast upon the coincidence of the cardinal planes of the limb and the planes of the splits carried out in the present work.

[20] Tornier ('96) found that two digits could be produced if one digit of an adult salamander was bisected longitudinally with sharp scissors; if the cut was not exactly median, however, one portion of the digit became necrotic and did not grow.

From these experiments it is evident that a considerable degree of differentiation is possessed by the limb at stage 38. Numerous irregularities are observed, of which the greater number involve the radial side of the limb. No relationship between the plane of the split and the plane of symmetry can be established by these experiments.

GENERAL DISCUSSION

As pointed out by Harrison ('21), the problem of reduplication presents two more or less separate phases, "that of the division or repetition of parts and that of symmetry." He has discussed the question of symmetry and has treated to a lesser extent the phenomenon of division.

The fundamental cellular changes which result in the production of supernumerary parts are still beyond our reach, and this study adds nothing, except in a negative way, to this part of the problem. It has been seen that such details of cell arrangement and activity as are visible at different stages in the process of reduplication give no clue to the causes of doubling. In short, we are forced to the indefinite and unsatisfactory conclusion that a disturbance of nearly any kind, if applied to a limb bud before it becomes differentiated, may bring about reduplication.

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[21] It is not absolutely certain that the limbs in this operation were formed directly from the separated parts of the original bud.

III. Relations of symmetry in spontaneous reduplications

Leaving out of consideration for the present the limbs derived from splitting the embryonic rudiment, we find that reduplicating limbs in general fall into one of several groups: 1) A single extra member may be present on the radial or the ulnar side of the primary. 2) Two supernumerary limbs may be present; if so, both may be on the same side, or one on each side of the primary. The type of triplicate appendage in which the primary is the middle member may correspond superficially with the other, but as both the extra members are mirrored with the same limb they themselves are of like asymmetry, and thus do not, in the true sense of the word, comprise an extra 'pair' of limbs. All such cases conform to Harrison's modifications of Bateson's rules (p. 420). In no case that has been examined has a tertiary limb formed on an ulnar reduplicant. This may be accounted for to some extent by the small number of ulnar reduplicants, but it seems more probable that the tardy appearance of such secondary limbs is chiefly responsible, differentiation having become so far advanced by the time they appear that further reduplication is impossible.

Ubisch ('22, '23) holds that the region of reduplication is the second most favorable place for differentiation, while the primary limb develops from the most favorable. This conclusion is based on his conception that the limb-forming cells are arranged concentrically, according to the degree of their limb-forming potencies, and that there is a differentiation gradient (Differenzierungsgefälle) running from head to tail in the embryo as a whole.

In a series of publications Gräper ('22, '23, '24) has presented the results of his experiments in transplanting hind-limb buds in Anura. Since in most of the reduplicated limbs the extra bud developed on the fibular side of the original, this author concludes that differentiation of the rudiment begins on the tibial side and proceeds toward the fibular. If buds are transplanted at a stage when this polarization has progressed approximately half-way ('Tibial beginnende Querschnitts-Determination') the polarized half is no longer affected by the body, and the limb formed thus retains its original prospective asymmetry, while the undifferentiated half forms a harmonic member under the influence of factors from the body.

As an aid in the explanation of Dreifachbildungen, which he considers to be different from the paired reduplications (Fächer), Gräper suggests the following interaction between body and limb. If the 'Querschnitts-Organization' of a normal or an orthotopically transplanted limb corresponds with the 'Querschnitts-Organization' which normally sends factors into the bud from the body, then these factors work together in the growing limb and it develops only a single harmonic limb and never a further regenerate. However, if these 'Querschnitts-Organizationen' do not correspond, then the factors going out under the influence of the body effect a reversal in the transplant and there develops usually a simple harmonic extremity without a secondary regenerate. Or if this reversal is impossible against the strong fixation of the 'Querschnitts-Organization' in the transplant, then the latter grows as such and the factors from the body seek a new field of work and form a harmonic regenerate. 'Bruchdreifachbildungen' are thus formed by a break in the normal correlations. If the break is healed, the correlations are again in force. But if union is prevented (della Valle), the bonds remain free and a harmonic regenerate grows from the body.

Gräper also states that a delay in growth of the graft may permit factors in the body to overcome those in the developing limb and thus determine its asymmetry as a harmonic member. It is not quite clear whether Gräper believes this change can be brought about after the two-dimensional polarization ('Querschnitts-Determination') has taken place. If the growth goes on rapidly, this influence has no time to act and the limb then retains its original asymmetry. Medium delay in growth may result in doubling ('Facherbildung'). However, this relation of the rapidity of growth to asymmetry and reduplication seems questionable. Certain of my transplantations carried out on older Amblystoma embryos and larvae (results as yet unpublished) show that after the limb becomes visible as a definite bud the processes of healing and preliminary growth are greatly retarded. Yet these grafted limbs hold their original asymmetry most tenaciously. Furthermore, such grafted buds reduplicate almost as freely as rudiments transplanted much earlier in embryonic life. Gräper also finds that extra limbs more commonly develop when the original is disharmonic, thus confirming Harrison's observations on grafts in normal location. The suggestion is made that when a harmonic limb is formed the 'bonds' between limb and body are all taken up. But when a disharmonic member results, bonds are free and may be taken up by one or two secondary harmonic 'regenerates.' The inference from Gräper's work is that the tibiofibular and the dorsoplantar axes of the anuran limb are determined at about the same time. Lack of exact information on this point and on many of the experimental orientations of the grafts makes the interpretation of Gräper's results rather difficult.

When the proximodistal axis of the limb was reversed (reverse transplantation), Gräper found that a secondary regenerate formed only when the primary regenerate from the original proximal end was disharmonic (homopleural grafts).

Przibram ('24) interprets the primary disharmonic limbs described by Harrison in terms of 'proximal regenerates,' and the secondary reduplications as normal growth from the original 'distal end' of the bud. As Harrison points out in his most recent paper (p. 458, footnote), this interpretation is based on a misconception of the arrangement of axes in the embryonic rudiment. The anteroposterior axis of the limb disc in the embryo does not in any sense correspond to the proximodistal axis of the definitive limb. This is clearly shown by the results of all grafts which alter the orientation of this axis in Amblystoma. It is at present impossible to show that the reduplications formed after grafting experiments on the embryo correspond, in manner of formation, to those produced by superregeneration of the injured definitive limb ('Bruchdreifachbildungen').

In nearly all cases the extra appendages stand in mirrored relations with the next adjoining, but there is a small group which contains animals having two limbs arranged in series. These exceptional cases have been commented on more fully elsewhere (Swett, '24), and only brief mention of them at this time is necessary. Obviously, some of these cases of serial arrangement may be explained on the basis of independent origin and development.[22] Others have developed under conditions so well controlled as to leave small doubt that they arose from the rudiment in exactly the same manner as the symmetrical pairs. Yet their components are of the same asymmetry.

We have seen already (p. 426) the manner in which this phenomenon may be brought about and the important rôle which complete or partial recovery of posture by rotation plays in the process. A primary limb, developing from an inverted rudiment, may rotate sufficiently toward its normal posture to retain its original asymmetry even when a reduplicating member is present. In such event, both limbs are of the same asymmetry, provided the reduplicant begins its development before rotation of the primary limb takes place, and subsequently becomes separated completely enough from the primary not to be involved in the process of rotation. This is subject to qualification to the extent that a direct relation must be established between the reduplicant and the body wall of the animal, inasmuch as differentiation of the dorsoventral axis of the extra limb, like the corresponding axis of the primary member, is determined by the tissue immediately surrounding its base. This fact may cause the reduplicant to be inverted in case the primary limb is upside down and no direct connection between the supernumerary limb and the body wall occurs. We thus find an explanation for certain types of exceptions to the rules of minor symmetry. Furthermore, in following the development of these cases, certain clues are given us concerning the determination of asymmetry in the reduplicant, and we may be permitted to conclude that: 1) Polarization of the anteroposterior axis of a secondary bud takes place in a direction opposite to that of the anteroposterior axis of the primary bud. 2) Reversal of this axis takes place very early in development, probably at the time the secondary growth center is formed. 3) In subsequent development this axis remains constant. 4) The dorsoventral axis of a reduplicant is determined by its relationship to the tissue immediately surrounding its base. 5) This occurs at a later time than the determination of the anteroposterior axis.

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[22] Harrison ('18, '21) reports three such cases. Two of them resulted from experiments in which the anterior half of the limb rudiment was removed. In each case of these, two limbs were formed, one from the remaining posterior half of the rudiment, while the other probably regenerated from the anterior border of the wound. Both operations were performed on the left side of the body. In one case the posterior member was defective, so that it was impossible to tell whether it was a right or a left, but in the other both limbs were definitely lefts. The third case resulted from the substitution of the dorsal half of a left rudiment for the dorsal half of a right. Two right limbs formed, one from the transplant and the other probably from the stationary ventral half. The normal posture of the dorsal limb (disharmonic limb) was regained by rotation.

IV. Relations of symmetry in artificially divided limb buds

Several investigators[23] have produced limb duplicities by a direct artificial division of one growth center into two. In nearly all the cases reported two limbs in mirrored relations resulted from the split.[24] In some instances it is difficult to prove that these developed from the growth centers formed by halving the original, but in others such is undoubtedly the case. Evidence furnished by the present study enables one now to suggest an interpretation of several groups of these previous experiments.

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[23] Compare Tornier ('97, '05), Przibram ('02), Reed ('04), Zeleny ('05), Megusar ('07), Lissitzky ('10), Fritsch ('11), Kurz ('12), and Detwiler ('22).

[24] Exceptions may be made in the case of Tornier ('05) and Lissitzky, whose experiments typically gave rise to three limbs; Detwiler, who reports two limbs

The salient facts brought out concerning the divided limb rudiments in the present investigation are: 1) the two primary limbs derived one from each isolated portion of a single split rudiment are always of like asymmetry and, 2) certain ill-defined spheres of influence exist, within which one primary limb apparently responds in certain definite ways to the presence of the other. The limb complex may consist of: 1) two single primary limbs of like asymmetry; 2) two primary limbs, both of the same side, one of which is reduplicated on the border nearer the other; or possibly, 3) two limbs of opposite asymmetry (v. infra).

No individuals exemplifying the serial arrangement of the two single limbs appear in the present series, but Detwiler's case (p. 440, footnote) is a diagrammatic example. If the two primary limbs develop far enough apart, so that there is no opportunity for mutual interaction, they will retain their original asymmetry. In such cases the limbs develop from half buds exactly as if a portion of the rudiment had been transplanted to a heterotopic location (Harrison, '18).

Symmetrical doubling of one of the members occurred in nearly every case not only when both of the primary limbs survived, but also in about half of the cases when one was resorbed. These cases are analogous to those in which a limb, transplanted to a position immediately behind the normal, reduplicated under the influence of the latter (compare Detwiler, '20). What the nature of the influence is or how

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of the same asymmetry, and possibly also in the one case given by Fritsch (p. 463 et seq. and figs. T′ and U′). This latter case consists of two limbs diverging at the elbow which arose from the 'T-Schnitt' operation of Barfurth. No mention is made of the asymmetry of the more anterior of these limbs, though it has the posture of a harmonic member. Figure U′ shows definitely that the posterior member is harmonic.

it is transmitted from one limb to the other are questions to which no answer can be given at this time.[25]

The triplicate limbs resulting from the experiments of Tornier ('05) and Lissitzky ('10) are entirely similar in arrangement to those produced in the present study. In the complexes reported by these investigators the normal limb and half pelvis which formed from the portion of the bud ventral to the split are analogous to the anterior (or ventral) primary limb and girdle formed in my series. The symmetrical pair of limbs and girdle 'regenerated' by the isolated dorsal part of the original bud corresponds to my posterior (or dorsal) primary limb with its reduplicant. The most important difference in the end results in the two groups of experiments involves the amount of girdle tissue formed. Tornier holds that each half of the dorsally isolated ilium regenerated the remainder of a half pelvis and a whole limb, and that the normal pelvis and limb regenerated from the portion of the bud which remained in its original position. If it were shown that the pelvic girdles with which these investigators worked constituted an equipotential system at the time of operation, the triplicate formations obtained would seem to involve the process of regeneration to a lesser extent than those of regulation and reduplication. Examination of the developmental stages would probably aid in arriving at the correct interpretation of these cases.

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[25] Wilhelmi ('20 a) has shown that doubling in the fluke or shank of the anchors found in Holothurians may be due either to a division of a single center or to a fusion of two. This is also true of the limb of Amblystoma, although, as has been shown, the relationships between the components thus formed may be very different in the two cases. In the event of a double anchor formed by the fusion of two anlagen, a stimulus from the parts on one side caused those on the other to form as their mirror images ('20 b). This dependent differentiation is distinct from the one which institutes the formation of the reduplicated structure. In the limb, differentiation of reduplicated parts is dependent not only on the 'influence' of the primary limb, but also on the relationship the secondary bud bears to the tissue surrounding its base. The nature of the 'influence' or 'dependent differentiation' involved is as difficult to determine in one case as in the other.

The duplicate and triplicate genital valves in intersexes of Lymantria, which Goldschmidt ('21) has described, are also interesting in this connection. If only two of these valves are present on the same side, both are of like asymmetry; but if another is formed between the first two, its asymmetry is reversed, thus furnishing another parallel to the arrangement of the limbs which develop from split rudiments in Amblystoma.

When two limbs of like asymmetry stand in series, marked reduction of one member often takes place. All three of the cases reported by Harrison (p. 463, footnote) show this phenomenon and figure 45 shows another such instance. In some cases there seems to be an incompatibility between two such limbs, but in all probability reduction is most often, if not always, due to the disadvantageous location of the weaker limb with reference to the blood stream. An investigation of the vascular connections within such complexes might throw some light on this phase of the question.

There remain for consideration those cases in which one of the limbs derived from a split rudiment is harmonic and the other disharmonic. In none of the numerous cases in the literature are developmental stages given and there is no positive proof that both the limbs resulting are actually primary. However, assumption of symmetrical relations between two primary components apparently does occur, although it is by no means clear how simple cleavage of the growth center could reverse the asymmetry of either component. We have here exactly the same problem which Spemann ('19) has subjected to such a thorough analysis in his discussion of the occurrence of situs inversus viscerum in one member of a pair of twins. Spemann does not consider that the operation could have caused the reversal directly and believes that it may be due to another factor entirely. Whatever this other factor may be, it, or one which acts in a similar manner, must be postulated to account for reversed asymmetry in one of two primary limbs. There is a strong probability that the process is akin to spontaneous reduplication and that the shock sustained at the time of operation sets in motion some unknown secondary factor which brings about the reversal. It is not constant in its occurrence, as the large number of normal twins in Spemann's experiments and the few exceptions to the rules of minor symmetry show.

GENERAL SUMMARY AND CONCLUSIONS

1. The double limbs on which the results given below are based have been produced by the following methods:

Transplantation of the limb rudiment of Amblystoma to a normal location in another animal, the grafted bud being from the same side of the body and implanted in inverted orientation or from the other side of the body and implanted in normal orientation with respect to its dorsoventral axis.

Division of the limb rudiment before it is visible externally or division of the early embryonic bud after it becomes visible. The halves of the split rudiments were kept apart by transplantation of a barrier of indifferent tissue into the wound.

I. Transplanted limbs

2. Reduplications occur in over 90 per cent of the cases in the above two series of disharmonic transplantations. They are of all types and grades. The reduplicating bud may usually be recognized early by reason of its massive size and its almost immediate separation into primary and secondary divisions. In some cases a third bud (tertiary) develops in connection with the primary or more rarely with the secondary limb. Occasionally, the primary and secondary (rarely also the tertiary) appear simultaneously. Radial reduplicants on the average appear several days earlier than do those on the ulnar side.

3. Double limbs mirrored in the radial plane are by far the most numerous; those mirrored in the ulnar plane are second, while dorsal and palmar mirror planes are usually secondarily assumed.

4. Exceptions to the rules of minor symmetry may be due, under some conditions, to a combination of the effects of reduplication and of rotation.

5. The polarity of the anteroposterior axis of a supernumerary bud is irreversibly determined at the time of its formation as the mirror image of the corresponding axis of its primary limb. The polarity of the dorsoventral axis of the reduplicant is determined at a later time in relation to the tissue immediately surrounding its base.

6. No clue as to the cause of reduplication is afforded by the microscopical study of a graded series of limb buds in the process of development.

7. Girdles of double limbs formed in normal location tend to be doubled. The doubling exhibited by the skeleton is not necessarily proportional to the amount of external doubling.

8. The musculature tends to be reduplicated in proportion to the reduplication of the cartilaginous part with which it is associated.

9. Complete peripheral innervation of double limb systems in normal location is accomplished by branches of the brachial plexus. If the components are rather widely separated, the second spinal nerve may join in the plexus formation.

II. Divided limb buds

10. The first (primary) limb which arises from each portion of a rudiment split in an early stage is invariably harmonic. The direction of the split is immaterial so far as the free limb is concerned.

11. When both primary limbs develop, the less normally located almost invariably forms a mirror-image reduplication on the side nearer the other. The single primary limb is the functional appendage in each case.

12. When the rudiment is split dorsoventrally, the anterior primary limb articulates with a girdle which is practically normal except for a reduction in the size of the coracoid. The posterior complex articulates with a small coracoidal plate or with no girdle tissue at all. When the rudiment is

split anteroposteriorly, the ventral primary limb articulates with the more normal girdle, the reduction being limited to the scapula. If the dorsal complex articulates with the girdle at all, a scapular element is the one involved. This is usually fused anteriorly with the scapula of the ventral limb.

13. Single limbs resulting from the posterior (or the dorsal) half of the rudiment, the other being blocked by the transplant, are usually practically normal. Single limbs resulting from the anterior (or the ventral) half of the rudiment develop very slowly and to about half normal size. The development of the girdle corresponds in general to the size of the limb. The free limb may be blocked completely.

14. The musculature and innervation of these limbs differ in no essential way from the conditions observed in the spontaneously reduplicated limbs (p. 469, 8 and 9).

15. Limb buds split after they become visible result in a large number of irregular appendages and a few double limbs. The irregularities are chiefly of two sorts—abnormal radial digits and supernumerary digits. Two cases with double limbs were produced whose components are irregular, although in each case one is reversed. One exception to the rules of minor symmetry has been found. Unless the bud is split into very nearly equal parts, the smaller atrophies or fuses with the larger.

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STUDIES ON THE CONTRACTILE VACUOLE OF AMOEBA PROTEUS[1]

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FOUR FIGURES

CONTENTS

Introduction 96
Material and methods 98
  A. Description of amoeba 98
  B. Culture methods 99
  C. Experimental procedure 100
Results 103
  A. The contractile vacuole in normal amoebae 103
    1. General description 103
    2. Relation of size of contractile vacuole to size of nucleus 106
    3. Relation of size of contractile vacuole to rate of contraction 107
    4. Relation of size of amoeba to amount of liquid excreted 109
  B. The contractile vacuole in portions of amoebae 114
    1. Manner of regeneration of new vacuole 114
    2. Time of regeneration of new vacuole 115
    3. Size and rate of new vacuole 118
    4. Volume of liquid excreted by new vacuole 121
  C. The contractile vacuole in amoebae immediately before and after fission 123
    1. Description of fission process 123
    2. Volume of liquid excreted before and after fission 126
  D. The contractile vacuole in binucleate amoebae 128
    1. Formation of a binucleate amoeba 128
    2. Volume of liquid excreted by binucleate amoebae 129
Discussion 130
Summary 137
Literature cited 138

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INTRODUCTION

Although the contractile vacuole of the Protozoa has been the subject of extensive observation and speculation since it was first studied carefully by Spallanzani in 1776, it is nevertheless true that only within recent years have empirical data of value been presented which justify certain tentative conclusions in regard to its structure and function. Many diverse functions have been attributed to this organ, but at present it is generally maintained that its function is two-fold in that it serves as an excretory organ, including excretion of CO_2 as well as nitrogenous waste, and also as an osmotic regulatory organ.

That the contractile vacuole is eminently fitted as an organ of excretion was made clearly evident by the demonstration of Jennings in 1904 that the contents of the vacuole were discharged to the outside. The actual composition of the fluid contents, however, has not been shown up to the present time. On the other hand, physiological observations indicate that the contractile vacuole holds metabolic products, since conditions which influence the rate of metabolism, such as changes in temperature, also have an effect upon the activity of the vacuole.

The relation of the contractile vacuole to osmotic conditions is clearly indicated by the general absence of a contractile vacuole in marine protozoa. Still more convincing is the experimental evidence presented by Zuelzer ('07, '10) and Hogue ('23). The former, by the transfer of a fresh-water amoeba to salt water, caused the disappearance of the contractile vacuole; and the latter, performing the reciprocal experiment with a salt-water form, initiated the origin of new contractile vacuoles. It seems probable, then, that the presence of a contractile vacuole is an adaptation to life in fresh water where the primary function of the vacuole is one of regulation of osmotic relations with the environment, and the function of excretion is only secondary, since this organ is not necessary for excretion in the marine protozoa.

The structure of the contractile vacuole has been determined with some accuracy. Khainsky's work in 1910 showed that the pore opening to the outside was temporary, appearing only at systole, and the more recent experiments of Taylor ('23) and Howland ('24) demonstrated that the vacuolar 'wall' is a temporary membrane disappearing at systole and forming anew at each diastole. Concerning the activity of the contractile vacuole, on the other hand, there are two questions which are still the subject of much controversy. These are the ultimate origin of the vacuole and the mechanism of systole. The investigations of Khainsky ('10), Taylor ('23), and Howland ('24) indicate that certain granules, so-called Excretkörner, are the precursors of the contractile vacuole and that the origin of these granules is associated with the food vacuoles.

Although we have this rather credible theory of the origin of the vacuole, the mechanism of the systole is more obscure, even though many attempts have been made to describe the series of events which lead to and cause the contraction of the vacuole. The phenomenon was explained by Maupas ('83) as due to the contractility of protoplasm and by Gruber ('12) as due to the elasticity of protoplasm, while Bütschli ('83) and others considered the force of surface tension to be the cause of systole. More recently, Taylor ('23) has formulated a theory from his observations on the contractile vacuole of Euplotes. He stated that before systole the contractile vacuole approached the periphery of the organism and came in contact with a definite spot in the ectoplasm, the papilla pulsatoria, and at this point of contact both the gelated ectoplasmic boundary and the wall of the contractile vacuole reverted to a sol condition. This resulted in a temporary pore, and, due to intracytoplasmic pressure, the contents of the contractile vacuole was discharged to the outside.

Since there still exists some degree of uncertainty as to the function of the contractile vacuole as well as gaps in our knowledge of its general activity, in the present investigation, a different method of experimental approach to the problem was tried, in the hope that it might throw some light upon the significance of the contractile vacuole in the economy of the organism. In brief, the new method consisted in cutting the protozoon, Amoeba proteus, in such a way that a nucleated but non-vacuolated piece was obtained. It was then possible to observe the regeneration of a new vacuole in this portion, with special reference to the time, place, and manner of formation, and consequently to acquire important data in regard to the activity and characteristics of this new-formed vacuole. Furthermore, as the original amoebae varied in size, as also did the resulting portions, there was afforded the opportunity to study the relationship of the activity of the contractile vacuole to the amount of cytoplasm and nucleoplasm. In addition to the above points concerning the new contractile vacuole in the portions, the investigation necessarily also embraced an intensive study of the original contractile vacuole in normal amoebae. This section of the work included observations on the size and rate of pulsation of the vacuole and the relation of these two factors. In addition, determinations were made of the amount of excreted material in order to compare the amount with that excreted by the regenerated contractile vacuoles in the portions of amoebae.

I am glad to express my appreciation to Prof. Lorande L. Woodruff for suggesting this study and for his help and interest during the course of the work. I am also indebted to Prof. J. W. Buchanan for helpful criticism.

MATERIAL AND METHODS

A. Description of Amoeba

The organism used in this investigation is one commonly known as Amoeba proteus. However, in 1916, Schaeffer divided the species proteus, retaining the name proteus for the amoeba with discoidal nucleus and longitudinal ridges on the pseudopodia and giving the specific name discoides to another which also possesses a discoidal nucleus, but is without longitudinal ridges on the pseudopodia. To a third, called Amoeba proteus by Penard in 1905, which is characterized by an ovoid nucleus, Schaeffer assigned the specific name dubia. Although the animal under consideration in these pages has the characteristics of Amoeba dubia Schaeffer, the old name proteus will be retained.

When moving forward, this amoeba has an average length of 400 μ, and sends out a profusion of pseudopodia on all sides, in this latter respect differing from many of the larger amoebae. It possesses an ovoid nucleus with an average size of 31 by 37 μ, and there is usually one large contractile vacuole near the nucleus. The animal forms unusually large food cups and, being quite voracious, is often congested with food vacuoles. One of the distinguishing features of this amoeba, however, is the variety of shapes of crystals in the endoplasm. A type of crystal which is especially characteristic and readily recognized is rectangular, long and wide, but very thin like a pane of glass. The morphological details described above may be seen in figure 1.

B. Culture method

The original culture of amoebae was obtained from Dr. J. A. Dawson, of Harvard University, in the spring of 1924, and subcultures were made essentially according to his method. The contents of a small bottle was distributed among a half-dozen finger-bowls, to each of which was added a small quantity of spring water and two grains of wheat, boiled to destroy the germ. A little spring water was added from day to day until the bowls were full. The amoebae were carried on in this type of mass culture throughout the course of the experiments. The original culture contained small flagellates, ciliates, and algae, which were carried over with each transfer into new cultures and served as food for the amoebae. The first method used for beginning a new culture was as follows: Two grains of wheat were placed in a finger-bowl with more than enough spring water to cover the bottom of the dish, and then three or four pipettes of water containing amoebae with flagellates, ciliates, and algae from the old culture were added. Every two or three days after this small quantities of spring water were added until the bowl was full. A more convenient and equally satisfactory method of continuing the cultures was employed later which involved simply dividing an old culture in which the amoebae were growing luxuriantly. The new culture was started with one-half the fluid and wheat grains of the old culture, and enough spring water added to both the old and new culture to make up the volume, together with fresh grains of wheat. Both of these cultures would then usually remain in good condition for several weeks with no more attention except the addition of enough spring water to compensate for evaporation. The cultures were kept covered with glass plates and out of direct sunlight. The progress of the cultures could easily be followed directly under the binocular microscope, and even with the unaided eye it was possible to see the relative number of amoebae present in the culture, although the microscope was necessary to determine whether or not they were in a healthy condition. This one strain of amoeba, bred under identical conditions for nearly a year, has afforded an unfailing supply for all the experiments of the present study.

C. Experimental procedure

With an ordinary pipette an amoeba was isolated under the binocular dissecting microscope onto a plain slide and placed on a warm stage at 80°F. under the compound microscope. After the amoeba had been located and had become attached to the slide, a sufficient quantity of culture fluid was added to the slide to compensate for evaporation. About fifteen minutes were allowed to elapse before physiological observations were begun, in order to allow the fluid to attain the constant temperature, at which all the studies were made, and the amoeba to become adjusted to its surroundings. During this interval careful measurements of the nucleus were made with an ocular micrometer.

Observations were then started upon the contractile vacuole. For a half-hour or longer, the exact time at which the completion of each systole occurred was recorded with the aid of a stop-watch, and also the exact size of the contractile vacuole was measured just before the onset of each systole, that is, when it had reached its maximum diameter. This afforded an accurate record of the size and length of diastole of each successive contractile vacuole in a period of a half-hour. After the completion of these records a camera-lucida drawing of the amoeba was made.

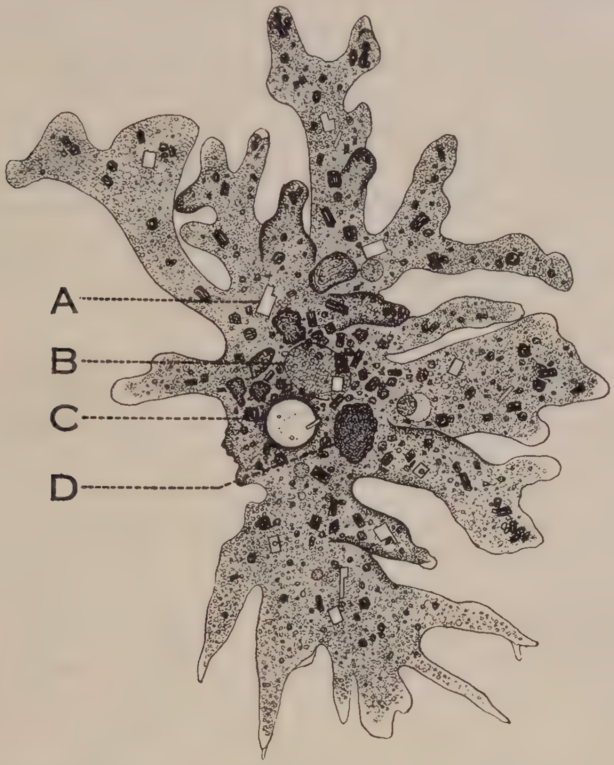

Fig. 1 Amoeba proteus (A. dubia Schaeffer). *A*, crystal; *B*, nucleus; *C*, contractile vacuole; *D*, food.

The next procedure was to cut off with the tip of a carefully sharpened 'iris knife-needle' a portion of the amoeba containing the contractile vacuole, leaving the nucleus in the other part. A difficulty presented at this juncture was the fact that usually the vacuole and nucleus remained closely associated most of the time, and frequently in such a relation that the contractile vacuole overlay the nucleus. Thus there was involved sometimes a wait of one-half hour or more for the two to move apart sufficiently to cut between them.

The result of the operation was two portions, the proportional size of which varied, depending upon the relative positions of the nucleus and contractile vacuole in the amoeba at the time when an opportunity for cutting presented itself. When the cut was made, a careful estimation of the proportional size of the two portions was recorded. If the piece with the nucleus was very nearly as large as the original amoeba it was called a 1– portion, and other estimates of the sizes of the nucleated portions were designated as ¾, ⅔, ½, ⅓, or ¼ portions as the case might be.

As has been said, one of the portions resulting from the operation contained the nucleus and the other the contractile vacuole, and it was the nucleated piece which was used for further observation. This portion was kept under the closest scrutiny from the time of cutting until a new contractile vacuole appeared, in order to observe the latter when it first formed and to record the time interval between the removal of the original and the formation of the new vacuole. When this new contractile vacuole had reached its maximum size, each diastolic interval and the diameter at the height of each systole were recorded during a half-hour period in the same manner as has been described for the original contractile vacuole. To complete the observations, camera-lucida drawings were made of the two portions of the amoeba.

It is important to emphasize at this point that during the entire time which one amoeba was under experimentation on the warm stage, which varied from three to four hours, the animal was normal in every way. It showed by its appearance, locomotion, capture of food, and even in some cases by the fission process that it was perfectly adapted to its environment, which had been changed practically in no respect from that of the stock culture except very slightly in temperature. A few times, for trial purposes, the amoeba was left on the stage for as long as six hours, drops of fresh culture fluid being added at intervals, and at the end of that time the animal was normal in every way. It is clear, then, that in the following observations we have to do with entirely normal phenomena.

RESULTS

A. The contractile vacuole in normal amoebae

1. General description. As has been stated in the description of this amoeba, there is usually one large contractile vacuole which generally remains in the vicinity of the nucleus, though often slightly nearer the posterior end of the animal. The study of this normal contractile vacuole under constant conditions showed some variations, the most evident of which were in size and in rate of pulsation. In the ninety normal amoebae studied, the size of the vacuole varied from 25 to 59 μ in diameter. Furthermore, even in a single animal within a half-hour the diameter of the vacuole was observed to vary from 34 to 50 μ under as nearly as possible constant conditions. On the other hand, in some amoebae there was very little difference in the size of the contractile vacuole within the half-hour or even none at all in one case. The average diameter of the contractile vacuole in seventy animals was found to be 36 μ.

In all the amoebae studied, the rate of contraction varied between five and ten systoles in a half-hour; and the time interval between any two systoles was found to vary from two to eight minutes, although the average length of all the diastoles in seventy amoebae was four minutes. In the same animal there were also differences in the length of diastole within a half-hour, the interval between two systoles varying from two minutes fifty-five seconds to seven minutes nineteen seconds. It was not usual, however, to have such a great difference, although a variation of two or three minutes was common.

Furthermore, the speed of systole itself was not constant in every case, although no actual measurements were made in this regard. As a rule, however, systole was instantaneous, but in some cases the emptying process seemed to be slowed somewhat for no apparent reason, while in a few instances the appearance was as if there was present some condition tending to prevent the normal process of systole. In this latter circumstance there was an unusual flow of cytoplasm toward the vacuole which was compressed thereby, and under these conditions the vacuolar fluid was partly or entirely expelled. In other cases the vacuole, having been partially emptied, filled, and again partly emptied several times in succession.

The contractile vacuole in Amoeba proteus, as in many other Protozoa, is formed from several small contributory vacuoles which gradually unite. At times an extra vacuole was seen to arise from the failure of a contributory vacuole to become confluent. In some cases this was due to the isolation of a contributory vacuole by the intrusion of a food mass between it and the primary vacuole, and at other times to a contributory vacuole being carried away by a current of cytoplasm. As a result of this separation, the two vacuoles sometimes would continue to function independently for a time, but usually sooner or later they approached each other and became confluent. It is highly interesting to note that these contributory vacuoles, therefore, are actual potential contractile vacuoles and can contract, which proves that the power of contraction is not peculiarly the property of the one primary contractile vacuole.

Rather significant in connection with the normal behavior of the contractile vacuole are some observations concerning movements of the protoplasm in its vicinity immediately preceding and following systole of the vacuole. Although the cytoplasmic behavior to be described was not observed at every systole, it was of rather frequent occurrence. A few seconds before systole there was noticed a streaming of cytoplasm, rather abruptly initiated, in a direction away from the contractile vacuole. In fact, during the course of the experiments, this movement quite often served as an indication of the approach of the time for systole. However, no sooner was this movement perceived than a flow occurred in the reverse direction, that is, toward the vacuole, which was followed almost instantaneously by systole. This last movement, if not actually initiating the process of emptying as it has the appearance of doing, certainly is associated in some way with the process of discharge of the vacuolar contents. It is probable that both movements, in fact, are the result of two rapid and succeeding changes in the adsorption phenomena associated with the vacuole-cytoplasm interface.

Another phenomenon which seemed to occur invariably in connection with the contraction of the vacuole is also of interest. Four or five seconds after systole the protoplasm of the amoeba in the vicinity of the discharge showed a positive tropism toward the fluid excreted by the vacuole. The appearance simulated a reaction toward a source of food, in that from one to three small pseudopodia were sent out into the region of the excreted liquid. These pseudopodia were very small, measuring about 25 μ, and were, as a rule, formed entirely of ectoplasm, so that it was difficult to see them unless the place of discharge of the vacuolar contents was on the 'edge' of the amoeba. About thirty-five seconds later, either they were withdrawn or the endoplasm flowed into them. Figure 2 shows sketches of the region of discharged vacuoles representing four different instances of the occurrence of this phenomenon. It is evident from these observations that the contractile vacuole discharges a substance to which the protoplasm of the amoeba is 'positively chemotactic.'

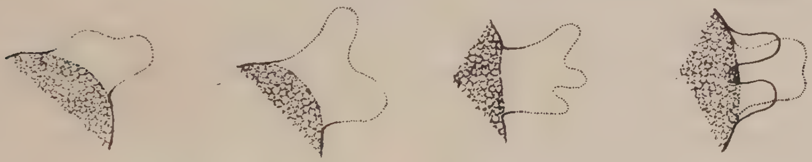

Fig. 2 Different instances showing the response of the protoplasm in the vicinity of a contractile vacuole a few seconds after systole.

2. Relation of size of contractile vacuole to size of nucleus. As the diameter of the vacuole varied remarkably among the different amoebae of this one species in the same culture and under the same conditions, it seemed important to determine if possible what factor or factors were involved in this variation. Since nuclear size was also a variable, computations were made to ascertain if there was any relation between the size of the nucleus and the size of the vacuole.

The tabulated results obtained from fifty-three amoebae affording available data are shown in table 1. At the top of this table are indicated the nuclear dimensions, which range from 25 by 31 μ to 43 by 50 μ; while in columns under the corresponding nuclear size is given the size of the vacuole of each of the fifty-three amoebae. To determine for each amoeba the size of the vacuole, an average was taken of the diameter of all the vacuoles formed in half an hour. Then for all amoebae having a nucleus of the same dimensions, the average diameter of the contractile vacuole was recorded. As the table shows, these range from 30 μ, in the amoeba which has next to the smallest nucleus, to 50 μ in the amoeba with next to the largest nucleus. Inspection of these averages show that there is a general increase in the diameter of the vacuole, as the nuclear dimensions increase, although there are slight fluctuations.

These irregularities are explainable by the fact that in some cases only one amoeba was studied for one size of nucleus, and also by the fact that some of the nuclear measurements were only very slightly different. By grouping those nuclear sizes which were nearly the same, all fluctuations disappear, as shown at the bottom of table 1. Grouping the amoebae according to nuclear size, the vacuolar diameter shows a progressive increase from 31 μ for the amoebae with the smallest nuclei to 33.1 in the next group, to 37.6, to 40.8, and finally to 42.4 μ in the group of animals having the largest-sized nuclei. These results show definite evidence, therefore, that amoebae with the smallest nuclei have the smallest contractile vacuoles and that with increase of nuclear size there is also a general increase in the size of the vacuole.

Furthermore, upon selecting from the culture several of the largest amoebae, it was found, upon measuring the nuclei in these animals, that they were very large, and conversely, in a selection of the smallest animals the nuclei were of small dimensions. We can say, therefore, that, in general, the smaller the nucleus the smaller the size of the amoeba; and hence the smaller the animal, as judged by nuclear size, the smaller the contractile vacuole. This relationship, of course, is to be expected as the organism with less protoplasm would produce a smaller amount of waste material to be thrown off, but it is none the less important to place the relationship on a basis of observed fact which, I believe, the preceding data have done.

TABLE 1

Relation of size of contractile vacuole to size of nucleus

SIZE OF NUCLEUS	25×31	28×32	28×37	31×34	31×37	32×37	34×37	32×39	31×43	32×43	34×40	34×43	37×40	37×43	40×43	43×50
SIZE OF CONTRACTILE VACUOLE	31.2	30.0	33.8	32.8	30.8	41.1	41.1	38.5	38.1	38.0	35.0	39.1	42.7	48.1	50.0	46.0
	32.0			31.5	34.3	37.7	46.8		39.7		41.7	38.6	41.2	38.5		
				34.3	37.2	36.4	37.5		38.5		41.4	42.9	42.4			
					35.6		36.4		53.1		42.1	34.7				
					38.8		37.5					45.5				
					37.2		37.5									
					35.2											
					38.2											
					40.3											
					33.6											
					32.3											
					36.6											
					35.7											
					44.2											
					39.5											
Average size of contractile vacuole	31.6	30.0	33.8	32.8	36.6	38.4	39.4	38.5	42.3	38.0	40.0	40.1	42.1	43.3	50.0	46.0
Average nuclear sizes grouped	31.0		33.1		37.6				40.8			42.4				
					36.9		39.3					42.1				46.0

3. *Relation of size of contractile vacuole to rate of contraction.* In order to determine whether there was any relation

between the size of the vacuole and the rate at which it contracted, these two factors were analyzed (table 2). Observations taken for the length of a half-hour from sixty-four different amoebae were used in this tabulation. For each amoeba the length of diastole of each contractile vacuole was recorded in a column corresponding to its diameter immediately before systole. If within the half-hour there occurred two or more vacuoles of the same diameter the average length of diastole for these was recorded. For each size of vacuole, then, the average length of diastole in the sixty-four amoebae was computed. The averages thus obtained show that, in general, the longer the interval between two systoles, the larger the contractile vacuole, or, in other words, the faster the rate of pulsation, the smaller the contractile vacuole. For the smallest size of contractile vacuole which measured 25 μ in diameter the average length of diastole was two minutes twenty-seven seconds, while in the case of the largest vacuoles measuring 56 μ the length of diastole was eight minutes sixteen seconds. The contractile vacuoles between these two extremes of size showed the same relation of the diameter to the length of diastole; that is, from the smallest vacuole to the largest there is a gradual increase in the average length of the interval from systole to systole. There are a few irregularities, but when the vacuoles with only very slight differences in diameter are grouped together, these fluctuations disappear, as indicated at the bottom of table 2.

Figure 3 gives a graphic representation of this relationship. On the abscissae are plotted the vacuolar diameters, while the length of diastole is plotted on the ordinates. It can be clearly seen that as the time of diastole lengthens the size of the vacuole increases.

The results shown here indicate that there is a continual and more or less constant accumulation of liquid in the cytoplasm, because when systole was delayed the contractile vacuole continued to grow larger with the collecting fluid. Furthermore, this accumulation of liquid being constant, one would expect a certain unit increase in diameter of the vacuole to take longer as the vacuole continues to grow larger, because of the volume-diameter relations. This is actually found to be the case, for in the vacuoles measuring from 25 to 40.6 μ in diameter it took an average of twenty seconds for the vacuole to increase 3 μ in diameter, while in the case of vacuoles measuring from 40 to 56 μ the same increase in diameter took forty-nine seconds.

The variability in the size of the contractile vacuoles is suggestive in connection with the phenomenon of systole, and in considering this process later its significance will be discussed more fully.

4. Relation of size of amoeba to amount of liquid excreted. Because the size of the contractile vacuole and its rate of pulsation were both such variable factors in this amoeba, neither one could be used as a criterion of the amount of liquid excreted by the animal. Consequently, to make comparisons of the amount of excretion in the individual amoebae it was necessary to compute the actual volume of liquid expelled from the contractile vacuole. The two factors necessary for the computation are, of course, the size of the contractile vacuole and the number of its pulsations within a definite unit of time. As the size of the vacuole varied in the same animal, the average size in the half-hour observation period was used, computing the volume of a vacuole of such a size and multiplying this result by the number of contractions of the vacuole in the half-hour. In this way a close approximation to the amount of liquid excreted by an amoeba during a period of one-half hour was secured.

The results obtained in this way from fifty-three different amoebae appear in table 3, which shows that the volume of liquid excreted in a half-hour varied from 98,286 to 458,150 cu. μ. It can be seen from the averages that the amount of liquid excreted from the vacuole is related to the size of the nucleus; that is, the amoebae with a smaller nucleus and hence, in general, with a smaller cytosome excreted less than the larger amoebae. Grouping together amoebae having nu-

TABLE 2

Relation of size of contractile vacuole to length of diastole

	SIZE OF CONTRACTILE VACUOLE	25	26.5	28	29.6	31	32.8	34	35.9	37.5	39	40.6	42	43.7	45	46.8	48	50	53	56
Length of diastole	1							4'12"		4'14"	3'50"	4'19"	6' 1"	6'21"	8' 9"					
	3					3'20"	2'50"	3'12"		4'66"	4'40"									
	4							3'20"	2'55"	4'	4'25"	4'38"	4'58"	7'22"			6'43"	11'43"		
	5									3'		4'12"	5' 9"	4'41"						
	6					2'42"	2'53"	3'33"												
	7									3'22"			5'23"	4'50"	7'19"	6'28"	5'38"	7'		
	8	2'32"	2'46"	3'12"	4'49"	3'26"	3'38"	3'47"												
	9					2'45"		3'50"	4' 3"	4'										
	10										2'16"	3'13"	3'32"	3'23"		3'22"				
	11	2'18"		3'30"		3'40"	5' 6"	5'29												
	12							2'43"	2'59"	3'44"			4'33"	5'21"						
	13									4'47"		5'34"								
	14									3'42"		5'29"		5'14"						
	15					4' 5"	3'46"	4'32"												
	16					2'50"	3'29"	3'49"	3'23"	4'13"										
	17									4' 8"	4'58"	5'		6'32"						
	18					4'12"	2'55"		2'64"				6' 8"	5'42"						
	19									3'59"	3'74"			5'41"	5'44"					
	20	2'45"		2'17"		4'38"		2'20"		2'57"	2'43"	3'42"	4'45"	5' 5"	7' 4"			7'20"		
	21													5' 5"	6'30"	8'33"		8'24"		
	22					2'31"		3'21"	3'49"	3'49"										
	23			2'13"	2'24"	3'15"		3'38"		4'39"										
	24									3'51"	4'13"			5'20"						
	25		2'35"	3'	2'15"	2'41"	2'54"			4'18"										
	26				3'18"	3'39"	4' 8"	5'13"	4'44"											
	27					3'23"		4' 8"	4' 8"	5' 1"	5'12"									
	28					2'50"				3'40"										
	28×		2'10"	2'23"		2'54"		3'49"												
	29									4'35"		3'52"	3'62"	4'37"	5'54"	5'21"	5' 8"			
	30					2'13"	2'35"	3'13"	3' 7"	3'	3'44"	3'37"		3'34"		5' 8"	5'11"	4'39"		
	31					3'24"	3'49"	3'39"	3'19"					8'16"						
	32					2'38"		3' 2"		3'11"										
	33											4'14"		3'21"		4'36"		4'52"		

Length of diastole	34						2'57"		2'27"	4'	3'47"	4'20"	4'10"	6' 8"		5'26"				
	35			2'23"			3' 3"	2'46"	3'44"	2'56"										
	36							2'49"		4'	4'14"				4'58"					
	37						3'31"	3'38"	4'09"	4'41"										
	38					2'38"				3'20"		4'42"		5'40"						
	39					2'26"		2'36"		4'20"				4'35"		7'17"		6'47"	7'39"	
	40					2'40"		3' 1"	2'57"	4'45"										
	41	2'43"		2' 8"		2'52"	3'20"			4'15"	4' 3"	3'38"	3'11"	3'26"						
	42	1'57"				2'38"														
	43					3'		3'38"	3'13"	3'39"		4'39"		6'18"						
	44					3' 5"				3'46"		3'52"	5'11"	6' 7"						
	45							5'31"		4'37"	4'52"	4'42"								
	46									3' 3"		2'57"	3'25"	4'31"	3'18"					
	47					3'				5' 8"		4'22"		5'31"						
	48					2'30"		2'59"		3'21"				4'47"						
	49					3' 4"		2'49"		3'32"										
	50			3' 6"		3'22"		4'44"												
	51					2'50"		3'17"		5' 4"										
	52													4'51"				5'54"	4'30"	8'16"
	53							4'44"		3'54"		4'28"		5'27"		8' 5"				
	54					3'58"		6'20"												
	55		2'58"							3'26"				4'31"				5'38"		
	56									2'59"		4' 7"		4'19"		4'41"	3' 6"	6'22"		
	57							1'56"						4'43"						
	58					2'		2'27"		2'43"										
	59					2'20"		2'18"		2'48"	2'52"	2'45"	3'37"	4'20"						
	60					2'18"		2'44"		1'51"		2'45"		3'53"		4'47"	4' 6"	5'32"		
	61									4'12"	4'10"	4'								
	62					2'30"	2'33"	2'56"	3' 7"	3'50"										
	63									3'42"		4'45"	3'37"	5' 1"						
	64		3' 1"		2'56"	2'28"	3'45"	2'39"		3' 2"										
Average length of diastole		2'27"	2'42"	2'41"	3' 8"	2'49"	3'50"	3'32"	3'26"	3'48"	4'	4'10"	4'31"	5' 2"	6' 7"	5'41"	5'	6'34"	6' 4"	8'16"
		2'34"		2'41"	3'15"			3'32"	3'44"			4'10"	5'13"			5'41"	5'47"		6' 4"	8'16"

clei of very nearly the same size, and averaging the volumes of each group, this relation of amount of excretion to size of cytosome shows still more clearly. From an average of 119,842 cu. μ in the group with the smallest nuclei there is a constant increase to an average of 411,576 cu. μ in the group with the largest nuclei.

Thus, it can be stated definitely, that, in general, the larger the amoeba the greater the quantity of fluid expelled from the contractile vacuole. An interesting question to be raised here is whether the amoebae excrete amounts of fluid strictly proportional to their protoplasmic volumes. This cannot be investigated at present because of the lack of a method for calculating accurately the volume of an amoeba. But with a general conception of the proportional size of the amoebae represented in this table, and with a comparatively accurate knowledge of the amount of liquid excreted by them, the opinion formed is that the smaller amoebae excrete more in

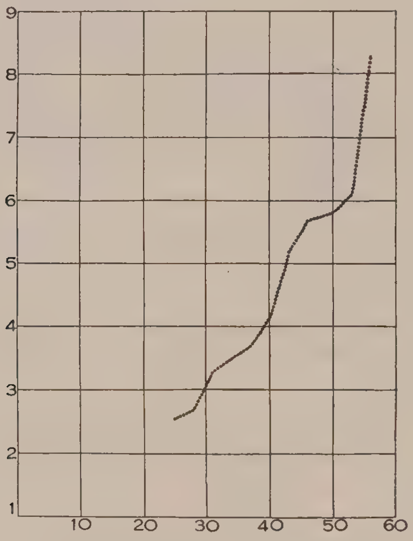

Fig. 3 Relation of size of contractile vacuole to length of diastole. The axis of abscissae represents the size of contractile vacuole in microns, and the axis of ordinates represents the length of diastole in minutes.

TABLE 3

Relation of size of nucleus to volume of liquid excreted by contractile vacuole

SIZE OF NUCLEUS	25×31	28×32	28×37	31×34	31×37	32×37	34×37	32×39	31×43	32×43	34×40	34×43	37×40	37×43	40×43	43×50
VOLUME OF LIQUID EXCRETED	127,832	141,372	182,807	129,477	130,744	327,739	237,612	269,759	203,108	201,444	157,738	204,882	244,725	350,135	458,150	365,008
	111,853			98,286	170,000	196,734	217,220		431,521	269,759	303,649	211,057	258,082	299,126		
				170,128	215,262	228,360	215,702				371,697	249,221	309,532			
					236,716		193,487				294,826	208,191				
					245,922		228,360					370,397				
					203,398		262,385									
					172,237		248,505									
					176,224											
					206,231											
					200,039											
					176,640											
					257,341											
					190,811											
					272,570											
					194,824											
AVERAGE VOLUME EXCRETED	119,842	141,372	182,807	132,630	203,265	250,944	227,609	269,759	317,314	235,601	281,977	248,749	270,779	324,630	458,150	365,008
	119,842	141,372	145,174		203,265	238,825			268,114					324,630	411,576	

proportion to their volume. The experimental work in the next section, however, will throw more light upon this question.

B. The contractile vacuole in portions of Amoebae

1. Manner of regeneration of new vacuole. After completing observations on the normal contractile vacuole, the amoeba was cut, as described above, to give portions with and without the contractile vacuole. The nucleated portion quite often assumed a limax form and progressed steadily forward for a time, in one case for ten minutes, but all finally resumed the characteristic form of the species. On the other hand, the enucleated piece rounded up almost immediately after the operation, and the pulsations of the vacuole became slower. The history of these enucleated fragments will be considered later.

In every case a contractile vacuole was regenerated in the nucleated portion. This new contractile vacuole at its first appearance, which varied from a few minutes to nearly an hour after the operation, was very minute, and was, as a rule, located in the posterior region of the animal. A few minutes after its formation, however, it usually moved up to the nucleus, and thereafter stayed in this vicinity as in the normal amoeba. Soon it began to pulsate, sometimes when the vacuole was only 12 μ in diameter, at other times not until it had reached a size of 25 μ or more. Having once contracted, the vacuole continued to pulsate periodically, growing larger between each systole until it reached its maximum size.

As the manner of formation of the new vacuole was not always the same, a few cases will be described in which it was possible to make unusually clear observations. Completely continuous observation of the vacuole was quite often not possible when the vacuole was small, because of the interference of food vacuoles or crystals. In this study no relationship was observed between the amount of food, on the one hand, and the time of appearance, size, and pulsation rate of the contractile vacuole, on the other hand.

In one case the new contractile vacuole was seen forty minutes after the operation. A minute later, two other very small ones were seen in different parts of the amoeba, and five minutes later, the three vacuoles having moved together had become confluent, forming one vacuole measuring 25 μ in diameter. After five minutes more, this vacuole, having increased to the size of 31 μ, contracted. In another nucleated portion five separate vacuoles arose which finally joined to form two, one measuring 43 μ and the other only 13 μ in diameter. These two vacuoles eventually united to form the one definitive contractile vacuole. In other cases, however, the two or three small contractile vacuoles did not come together so soon, but continued to function separately for an hour before coalescing, or were still functioning independently even after twenty-four hours.

On the other hand, there was not always more than one new vacuole formed. In one portion, six and one-half minutes after having been cut, an exceedingly small clear vacuole was observed at the posterior 'edge' of the amoeba. It measured not over 5 μ in diameter, and only after twenty-five minutes did it show an increase in size. However, thirty-four minutes after the operation it had moved up to the vicinity of the nucleus, and having reached a diameter of about 12 μ it contracted. Thereafter it continued to pulsate rhythmically about every three minutes. An hour and fifteen minutes after the operation the vacuole measured 31 μ.

From these representative cases the following general statements as to the behavior of the regenerating contractile vacuole are made clear. The salient points in its manner of formation are, 1) its small size when it first appeared after a varying interval of time in the posterior region of the portion, 2) its subsequent growth and movement to a location near the nucleus and, 3) at about the same time, while still very small, its initiation of rhythmical pulsation and, finally, 4) its further increase in size until it reached its maximum.

2. Time of regeneration of new vacuole. Having noted that the length of time varied for the regeneration of a vacuole in the nucleated portion, it was of interest to discover, if possible, what determined this time interval. As the portions cut were necessarily of varying proportions, it was thought probable that the size of the piece might be the underlying factor involved in the regeneration. Consequently, an attempt was made to determine if any relation existed between these two factors. The two right-hand columns in table 4 show the results of this comparison. The first column gives the relative size of the piece, while the second gives the average time for the regeneration of the vacuole in the corresponding portions. Upon examination of these results, one sees that the largest portions regenerated a new contractile vacuole in an average time of 21 min. 53 sec.; the ¾ portions in 22 min.; while the portions ½ and ⅓ the size of the original amoeba had a vacuole in about 18 min. In the case of the smallest pieces, the time interval was about 29 min. for the ⅓ portions, and 15 min. for the ¼ portions; while in two very small portions, much less than a quarter of the amoeba, a new vacuole appeared in 26½ min. No regularity in the results is visible here, and the differences, in any case, are not sufficient to be significant.

As there was found to be no relation between the size of the portion and the time of regeneration of a new vacuole, the next point to be decided was whether the size of the nucleus had any determining effect. Calculations which are presented in table 4 were then made. At the top of the table are the nuclear dimensions, and in columns under them the time of regeneration of the vacuole in pieces containing a nucleus of the corresponding size. Although there are many irregularities, the averages show a definite relationship between these two factors.

By dividing the experimental animals into three equal groups according to nuclear size, the relation shows very clearly. In group B, where the nuclei are of the more usual size, the appearance of the new contractile vacuole occurred after an average interval of 21 min. 31 sec. In group A, which represents animals with the smallest nuclei, the new

TABLE 4

Time of regeneration of the contractile vacuole in relation to the size of the portion and to the size of the nucleus

SIZE OF NUCLEUS	25 × 31	28 × 31	28 × 32	29 × 32	28 × 34	31 × 34	31 × 37	32 × 37	34 × 37	32 × 39	31 × 43	34 × 40	37 × 37	32 × 43	37 × 39	31 × 46	34 × 43	37 × 40	37 × 43	SIZE OF PORTION	AVERAGE TIME OF REGENERATION
TIME OF REGENERATION	31′		57′			2′	24′	14′40″	19′50″		16′35″			10′						1—	21′53″
	15′				27′		35′		30′						25′		4′	18′		$\frac{3}{4}$	22′
		21′40″					30′ 40′ 18′ 14′30″ 36′30″ 22′		15′			21′			8′30″	5′30″	3′			$\frac{2}{3}$	18′ 6″
		12′ 16′45″ 19′30″		5′30″	30′ 36′		14′30″ 36′30″ 9′ 25′	6′30″	20′25″			25′		18′					10′	$\frac{1}{2}$	18′58″
						8′	52′20″			27′										$\frac{1}{3}$	29′ 6″
									30′				23′					5′ 3′		$\frac{1}{4}$	15′15″
		26′															27′			0+	26′30″
	23′	19′11″	57′	31′	5′30″	5′	24′45″	10′35″	23′ 3″	27′	16′35″	23′	23′	14′	16′45″	5′30″	11′20″	8′40″	10′		
AVERAGE TIME OF REGENERATION	Group A, 24′47″					Group B, 21′31″					Group C_1, 18′23″					Group C_2, 9′26″					
											Group C, 13′54″										

vacuole appeared in an average time of 24 min. 47 sec. which is not very different from the interval in group B, but does show that a slightly longer period is necessary for regeneration. On the other hand, in group C, which contains portions with the largest nuclei, the average time of the appearance of the vacuole was 13 min. 54 sec., which shows a decided shortening in the time of regeneration and is unquestionably significant. This is still more evident if, for the purpose of analysis, group C is divided again into two divisions, those with the smaller nuclei group C_1, and those with the largest nuclei of all group C_2. The result from this division of the experimental animals shows that the portions with the largest nuclei were able to regenerate a contractile vacuole in an average time of 9 min. 26 sec., that is, in almost half the time of group C_1, which was 18 min. 23 sec. Finally, then, it is evident that the four groups of operated animals show with increase in size of nucleus a progressive shortening in the time necessary to form a new vacuole from 24 min. 47 sec., in those with the smallest nuclei, to 21 min. 31 sec. in the next group, to 18 min. 23 sec., and finally to 9 min. 26 sec. in the portions with the largest nuclei.

We can fairly conclude, then, from these results that with increase in size of the nucleus there is an increase in the speed with which a contractile vacuole can be regenerated in a portion, and that this increase is rather gradual until the nuclear size is at its maximum. Then there is evident a marked increase in the readiness with which a new vacuole can be regenerated.

3. Size and rate of new vacuole. When the regenerated vacuole in the portion had reached its maximum size, which occurred about an hour after the operation, a careful study of its behavior was made in the same manner as in the case of the original vacuole. It was observed continuously for one-half hour, each succeeding vacuole being measured at the height of diastole, and the length of the interval between systoles recorded. Often the cut portions showed irregularities in the behavior of the vacuole and had to be discarded.

The most common irregularity was the presence of extra vacuoles, and in this case the portion was of no value for further observation.

Significant differences from the original contractile vacuole are shown in the size and rate of pulsation of the regenerated contractile vacuole in the portions. The changes in size of vacuole will be considered first.

a. Change in size of new vacuole. From a superficial survey it was seen that some of the new vacuoles showed an increase in size over the original, while in others there was a decrease. When, however, the size of the vacuole was considered in connection with the size of the portion, a definite relationship seemed to hold (table 5). In the largest pieces, i.e., those very slightly smaller than the original whole, there was on the average the slight increase of 0.6 μ in the size of the contractile vacuole after the amoeba was cut. In the ¾ portions there was a still smaller average increase of only 0.29 μ, while the ⅔ portions showed a negligible average decrease of

TABLE 5

Size of regenerated vacuoles compared with size of original vacuoles

RELATIVE SIZE OF PORTION	1—	3/4	2/3	1/2	1/3	1/4
INCREASE IN DIAMETER OF VACUOLE	11.46 9.68	7.69 6.00 2.98	5.80 1.09 1.59 4.68	6.40 2.80 2.60	1.30	
TOTAL INCREASE	21.14	16.67	13.16	11.80	1.30	
DECREASE IN DIAMETER OF VACUOLE	0.10 8.90 9.10	12.90 2.30	2.80 4.20 6.87	11.60 2.80	6.63 10.20	9.00 1.40 5.30
TOTAL DECREASE	18.10	15.2	13.87	14.40	16.83	15.70
AVERAGE INCREASE	0.60	0.29				
AVERAGE DECREASE			0.10	0.52	5.16	5.23

0.1 μ, and the ½ portions an average decrease in size of 0.52 μ. Although all these differences are slight, on the other hand, in the ⅓ and ¼ portions there was quite a different result: an appreciable decrease of over 5 μ in the average diameter of the new contractile vacuole. Making a general survey of the averages in the different portions, it is evident that, although in the two largest portions there was a slight but actual increase in size of the vacuole over the original, nevertheless, the proportional size of the vacuole of the portion to the original vacuole decreased steadily from the largest portion to the smallest.

As for the actual change in size of the new vacuole, it is clear that in the 1- to ½ portions there was very little change, the 1- and ¾ portions increasing slightly, while the ⅔ and ½ portions showed a negligible decrease. On the contrary, in the smallest portions, the ⅓ and ¼, there was a significant decrease in the size of the vacuole in comparison with the original.

b. Change in rate of pulsation of new vacuole. In order to consider the change in rate of pulsation of the new vacuole as compared with the rate in the original amoeba, the average length of diastole of the vacuole was computed for each original amoeba and for the new vacuole of each portion. The change in rate of the new vacuole as compared with the original was recorded either as an increase or decrease in the length of diastole, as the case might be.

The averages for each portion computed from these records showed an increase in the length of diastole in portions of each size studied, although in a few single instances there were very slight decreases of only a few seconds (table 6). As in the case of the change in size, the greatest variation in the change in rate occurred between the larger portions, the 1- to ½, on the one hand, and the smaller portions, the ⅓ and ¼, on the other hand. Specifically, all four of the larger portions showed an increase in length of diastole of over a minute, while in the case of the two small portions there was an average increase in the length of diastole of only a few seconds.

It is interesting to note, then, that in the smaller portions the contractile vacuole decreased in size, but kept up nearly to the original rate of pulsation, while in the larger portions the converse was true, there being little change in size, but a definite slowing of the pulsation rate.

These results concerning the change in size and rate of contraction of the new vacuole as compared with the original are of interest in connection with some investigations of Gruber ('12), which will be considered later.

TABLE 6

Rate of regenerated vacuoles compared with rate of original vacuoles

RELATIVE SIZE OF PORTION	1—	3/4	2/3	1/2	1/3	1/4
INCREASE IN LENGTH OF DIASTOLE	1′ 55′ 3′ 5″ 2′ 9″	29′ 2′ 55″ 35″ 32″ 2′ 51″	42″ 36″ 3′ 28″ 1′ 2″ 39″ 1′ 53″	1′ 8″ 1′ 16″ 2′ 20″ 3′ 3″	24″ 30″	52″ 1′ 3″
TOTAL INCREASE	7′ 9″	7′ 22″	8′ 20″	7′ 47″	54″	1′ 55″
DECREASE IN LENGTH OF DIASTOLE	9″ 39″	20″	33″	27″	32″	22″
TOTAL DECREASE	48″	20″	33″	27″	32″	22′
AVERAGE INCREASE IN LENGTH OF DIASTOLE	1′ 16″	1′ 10″	1′ 6″	1′ 28″	7″	31″

4. *Volume of liquid excreted by new vacuole.* As the size of the amoeba has been shown to influence the amount of fluid excreted in normal animals, it was thought that a study of the volume of liquid excreted by parts of known proportions, especially when compared with the normal volume, would be of peculiar value in determining the importance of the amount of protoplasm in influencing the quantity of excretion from the vacuole. Consequently, for each nucleated portion the volume of liquid excreted in a half-hour was computed, and this compared with the volume excreted by the

whole amoeba in the same length of time. Thirty cases were used in this comparison, the results of which are shown in table 7. At the top of the table are indicated the various-sized portions, from the largest to the smallest. The unit used for a comparison of volumes is the per cent of liquid excreted by the portion as compared with the amount excreted by the whole animal. These percentages, then, are arranged in columns under the portion of corresponding size. An examination of the percentages for the largest pieces (1-), which are almost the size of the original amoeba, shows that in every case these portions excreted over 100 per cent, that is, they excreted more than the amoeba before it was cut.

TABLE 7

Comparison of size of portion with volume of liquid excreted

RELATIVE SIZE OF PORTION	1—	3/4	2/3	1/2	1/3	1/4
PER CENT OF LIQUID EXCRETED IN COMPARISON TO WHOLE AMOEBA	179	80	66	69	50	72
	115	124	74	52	44	83
	115	72	108	82	53	25
	133	133	92	57	39	33
		105	76			64
		93	46			44
			89			
AVERAGE PER CENT	135	101	78	65	46	53
EXPECTED PER CENT	100—	75	66	50	33	25
PER CENT OVER THE EXPECTED PER CENT	35+	34	18	30	39	112

The average amount excreted by these largest portions was 35 per cent more than that excreted by the original amoeba, which is a result hardly to be anticipated. In the case of the ¾ portions, the expectation was that they would excrete approximately 75 per cent of the amount of fluid; but, whereas some of them excreted less than the original amoeba, others excreted more, and only two out of six approximated the expectation. In fact, the average amount excreted by the ¾ portions was 1 per cent more, or about the same as the original amoeba. Furthermore, all the different-sized portions showed a decided increase over the expected per cent, as can be seen from the averages in the table. In the lowest line of table 7 is indicated this percentage of increase over the expected per cent. For example, the per cent of excretion expected by the ¼ portions was 25 per cent, whereas they excreted on the average of 53 per cent. This, then, is 112 per cent over the expectation. The per cent above the expectation from the largest to the smallest portions forms the following series: 35+, 34, 18, 30, 39, 112. This series shows, with the exception of the two largest portions, that as the pieces become smaller they excrete increasingly larger amounts in proportion to their size.

C. The contractile vacuole in amoebae immediately before and after fission

Four opportunities for the study of the effect of fission upon the behavior of the contractile vacuole were presented during the course of this work. In one case division occurred during a relatively short interval in which observations had been suspended and consequently actual fission was not seen, although a study of the contractile vacuole was made before and after the process. However, in the three remaining cases, besides obtaining a complete record of the behavior of the contractile vacuole before and after division, the entire process was observed, and in one instance camera-lucida drawings were made at short intervals throughout the process. As the observation of division of Amoeba proteus is rare, the process will first be briefly described.

1. Description of fission process. The first indication of the approach of fission was shown by a gradual drawing in of the pseudopodia, that is, new pseudopodia sent out were shorter than normally is the case. Figure 4 (A) shows a camera drawing of an amoeba at just this stage with short pseudopodia on all sides. There was also a tendency for the amoeba to become contracted into a small compass and for locomotion to diminish considerably, the short pseudopodia merely being thrust out and withdrawn. Fifty minutes later, as seen in (B), the amoeba contracted still more until it was approximately spherical with short blunt pseudopodia, and progression entirely ceased. The very short pseudopodia which were continually changing extended not only laterally, but from all over the surface of the amoeba, as is indicated to some extent in (B).

In the next stage of the process these rather regular short pseudopodia were withdrawn, while laterally the protoplasm spread out in a thin area, leaving a concentrated sphere of protoplasm in the center. Pseudopodia were formed from this outer zone and then a slight lengthening of the amoeba was evident, shown in (C), due to the increased formation of pseudopodia at two opposite regions of the animal. Furthermore, at these two opposite regions the concentrated area of cytoplasm was thinning out and becoming continuous with the thin outer zone. In (D) it is quite evident that the cytoplasm from the concentrated central zone was flowing out at either end where the thin area was increasing and sending out larger pseudopodia.

One minute later (E), the concentrated area has been drained away almost entirely, the organism has lengthened considerably because of this flowing in two opposite directions, and the indications of the two daughter amoebae can be seen with the small concentrated zone between them. After one more minute (F), the individuality of the daughters is very evident, the two being connected by a rather stout strand, the latter being all that remained of the original concentrated center of the sphere. The next part of the series (G, H) shows no new process but the continuous lengthening and narrowing of this connecting strand while the two daughter amoebae slowly moved apart by the active formation of pseudopodia. Then one section of the connecting strand became thinner, and finally, just thirty-two minutes after the sphere stage as represented in (B), the strand broke (I), and the two daughter amoebae continued to move apart. The essential points of this procedure, then, were the concentration of the protoplasm into a sphere when nuclear division took place and the subsequent active flowing of the protoplasm from this concentrated area in opposite directions until there resulted two masses of protoplasm connected by a strand. When this strand became very thin, it broke and the two daughter amoebae moved apart.

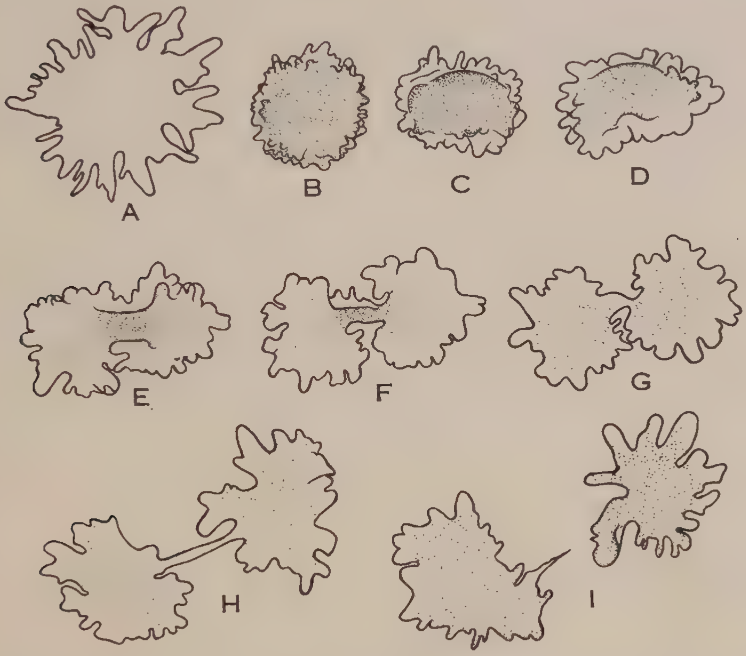

Fig. 4 Stages in the process of fission of Amoeba proteus. Time between each stage: A-B, 50 minutes; B-C, 19 minutes; C-D, 1 minute; D-E, 1 minute; E-F, 1 minute; F-G, 1 minute; G-H, 7 minutes; H-I, 2 minutes.

During the early part of the fission process when the amoeba was in a sphere, it was impossible to see the nucleus and the contractile vacuole, and it was therefore impossible to determine the origin of the two daughter contractile vacuoles. However, observations made as soon as possible did not reveal the presence of the original large contractile vacuole, but at the completion of division, or soon after, there was a small contractile vacuole in both daughter amoebae. As both these small vacuoles gradually grew larger in a similar manner to the regenerating vacuole it seems probable that they were both new contractile vacuoles.

Further evidence for the formation of new vacuoles was afforded by the observation of one amoeba in which small extra vacuoles appeared before division while the normal vacuole was of maximum size. It is probable that the old contractile vacuole disappears during the sphere stage and that two new vacuoles are formed, one in each daughter cell. There is no indication that the vacuole divides, as has been sometimes suggested. In fact, in one daughter amoeba under observation no vacuole was present until nearly an hour after the completion of division.

2. Volume of liquid excreted before and after fission. The study of the contractile vacuole just before division shows in three out of the four cases a relatively large amount of material excreted as represented in the second column of table 8. The rate and size of the contractile vacuole in all instances except one showed no unusual irregularities. In one amoeba, however, small extra vacuoles appeared before division, and this animal, amoeba no. 80 in table 8, showed an unusually high rate of excretion from the one main contractile vacuole in the half-hour preceding division.

The volume of liquid excreted by daughter amoebae was determined after division and the data are presented in table 8. In the first case, amoeba no. 7, the calculations were made one and one-half hours after the completion of fission, and the amount excreted by the daughter amoeba was 41 per cent of the amount that had been given off by the parent amoeba in the same length of time. In the next case, amoeba no. 41, the excretion by the daughter cell was studied three hours after division and found to be 49 per cent of the amount that had been excreted by the parent organism. Four days later, however, this daughter amoeba was excreting 80 per cent, the increase probably being due to growth during the four-day interval.

In the case of amoeba no. 77, both daughter amoebae were studied. One animal (no. 77X) was observed one hour after division and was found to excrete 81 per cent of the amount excreted by the parent, while the sister cell (no. 77Y) one and a half hours after fission was excreting 67 per cent. The time of appearance of the contractile vacuole may have been the reason for this difference in excretion, because in no. 77X there was a small vacuole immediately after division which later coalesced with other small ones, so that one hour after division the contractile vacuole measured 43 μ. On the other hand, in no. 77Y the contractile vacuole did not appear until nearly an hour after division. The observations of the origin of the contractile vacuole in these two sister cells also gives evidence for the statement that two new contractile vacuoles are formed, i.e., one in each daughter cell and the old contractile vacuole of the parent cell disappears. In the last case (amoeba no. 80) the daughter cell one-half hour after fission excreted 62 per cent of the amount excreted by the parent.

TABLE 8

Comparison of volume of liquid excreted by contractile vacuole before and after fission

AMOEBA NO.	AMOUNT EXCRETED BEFORE DIVISION	NO.	AMOUNT EXCRETED BY DAUGHTER AMOEBAE	PER CENT EXCRETED BY DAUGHTER	TIME INTERVAL FROM FISSION TO OBSERVATION
					hours
7	276,356		114,450	41	1½
41	327,739		161,716	49	3
77	309,532	77X	253,189	81	1
		77Y	209,727	67	1½
80	431,521		267,614	62	½
				60 average per cent excreted by daughter amoebae	

The average of these four cases of fission shows that the daughter amoeba soon after division excreted 60 per cent of the amount of liquid that the parent did. The ½ portions, as was shown earlier, excreted 65 per cent of the amount of the normal amoeba, so that the results from fission and from artificial division into two halves are not greatly different. In other words, the 'shock effect' was only 5 per cent. In brief, allowing for the considerable experimental error, it is still evident that half portions and daughter cells excrete significantly more than 50 per cent of the amount excreted by the whole animal.

D. The contractile vacuole in binucleate amoebae

Although only two binucleate amoebae were studied with reference to the effect upon the contractile vacuole, one of these offered the opportunity for the observation of the actual origin of the binucleate condition. It has been the opinion of some investigators that the binucleate condition is produced by the fusion of two uninucleate cells. However, Stolc ('06) observed that by altering the culture medium the binucleate form of Amoeba proteus could develop directly from the uninucleate condition, but he did not determine how this took place. Levy ('24), in an experimental study of reproduction in Amoeba proteus, concluded that polynucleate forms were probably due to failure of cytoplasmic division at the time of division of the nucleus, although he never observed this to happen under natural conditions.

1. Formation of a binucleate amoeba. The amoeba under observation went through the normal processes of fission as described above, up to the time when two daughter amoebae could be distinctly seen with only a strand connecting them, similar to the condition represented in figure 4, (G). The animal stayed in this state for a half-hour, while there arose in each part a small contractile vacuole. Then, for no evident reason, the protoplasm from one began to flow into the other, the final result being a binucleate amoeba, the nucleus having already divided, of course, when in the sphere stage. For a time after confluence of the protoplasm the two contractile vacuoles remained, but finally one disappeared. The two probably coalesced, although this was not actually observed.

This entire process in the formation of a binucleate amoeba was observed for the first time, it is believed, under normal conditions. Accordingly, it can now be stated definitely that, in some cases at least, binucleate amoebae are formed as the result of the interruption of cytoplasmic division after division of the nucleus has occurred.

2. *Volume of liquid excreted by binucleate amoebae.* In the amoeba described above the contractile vacuole was studied for the half-hour immediately preceding the rounding-up process, i.e., while uninucleate. It was very large, sometimes attaining a diameter of 59 μ, and the rate also kept up rather high, so that the amount of fluid excreted in the half-hour was 458,150 cu. μ, the largest amount recorded in this study. The next day, the behavior of the contractile vacuole in the binucleate amoeba was studied, and the amount of fluid excreted was still very high, but had decreased to 426,300 cu. μ.

On the next day, one nucleus had disappeared, and it was natural to suppose that the amoeba had divided, but there were no indications that it had done so, as the animal appeared as large as ever and there was no other amoeba on the slide. This disappearance of the extra nucleus had been observed before in another binucleate amoeba, so that all the available evidence indicates that the extra nucleus disintegrated in the cytoplasm. The amount excreted after the uninucleate condition was established was 207,360 cu. μ in half an hour, or less than half what it was before. On the next day, the amount excreted in a half-hour was 227,416 cu. μ, and by the following day, four days after the binucleate condition originated, the organism had divided. Its history was not observed further.

The other animal possessing two nuclei was first observed in the binucleate condition. Extra contractile vacuoles, either one or two, were present at different times and, due to this irregularity, the volume excreted was not computed. The next day one nucleus had disappeared, as was previously stated, and the contractile vacuole was unusually irregular as regards size and rate of pulsation. This amoeba was then used for the study of cut portions, and the ⅔ portion cut off with the nucleus showed no unusual irregularity.

Although only two cases of binuclearity were afforded for study, the observations indicated that the binucleate amoebae excreted a maximum volume from the contractile vacuole. Furthermore, after a uninucleate condition was resumed, as occurred in both cases without divisions of the animal, the volume excreted was less than half the former amount.

DISCUSSION

The method employed in this investigation involved cutting an amoeba in such a way that a study could be made of the behavior of a contractile vacuole regenerated by a nucleated non-vacuolated portion. Although the operative method has been used with amoeba by some authors, notably Hofer ('89), Stolc ('10), and Grosse-Allermann ('12) for the determination of the importance of the nucleus to the organism, and Gruber ('12) made some observations on the contractile vacuole in portions of amoebae, extensive operations have not been planned to study the regenerated vacuole.

Furthermore, in the present investigation intensive observations were made for a continuous period of one-half hour, and during this time each vacuole was measured at the height of diastole and the time between every two systoles was recorded. It should also be emphasized that the amoebae employed were from carefully controlled cultures, so that their immediate past history was the same in all cases—a factor of crucial importance for reliable results.

A striking fact obtained from the study of normal vacuoles was the rather great variation in size and rate of contraction of the vacuole under the same conditions not only as might be expected in different animals, but in the same animal within a short period of time.

It was demonstrated that among the different amoebae the size of the vacuole was, in general, dependent upon the size of the amoeba, the larger amoebae having the larger vacuoles. Taylor ('23), in his work with Euplotes, stated that the size of the vacuole tended to be proportional to the size of the organism. This relationship may be associated with the proportionally larger amount of waste material formed by a greater amount of protoplasm. However, the amount of increase in excretion is not directly indicated by the measure of increase in size of the vacuole because, as has been shown, the rate of pulsation decreased as the size of the vacuole increased.

This fact suggests an important point in regard to the influence of temperature on the contractile vacuole of amoebae. As is well known, a lowering of temperature slows the pulsation rate, and the natural conclusion drawn from this observation was that less material was excreted. However, a coincident increase in size of the contractile vacuole might suffice to keep up the actual amount excreted. At all events, the results of this study show that, in an investigation of the influence of temperature or in any other physiological experiments both factors, the size of the vacuole as well as the rate of pulsation, must be taken into consideration.

For example, in the case of the larger amoebae the increase in size of the vacuole entirely compensated for the decreased rate of pulsation, so that larger animals excreted actually larger amounts of fluid in spite of the lower rate of contraction of the vacuole. It is true, then, that a slight increase of a few microns in the diameter of a vacuole may entirely compensate for a decrease in the number of contractions.

Since we have seen that there is some correlation between the rate of pulsation and the size of the vacuole, it is pertinent to inquire if there is any causal relationship between the two. Does the rate of contraction determine the size of the vacuole, or is the converse true; or are they both, perhaps, the result of one or more other factors? The variation in size of the contractile vacuole in a single amoeba indicates that the process of systole is not dependent upon the vacuole's reaching a certain size, but that the stimulus for systole, whatever that stimulus may be, is able to cause contraction of the vacuole entirely independently of the volume of liquid that it contains.

This independence of the process of systole is still more clearly shown by the fact that the regenerating vacuoles begin to pulsate when they are very small, and also because small contributory vacuoles which have become separated from the main contractile vacuole will contract. This last fact regarding the pulsation of contributory vacuoles shows, in addition, that the ability to contract is not the property of the main contractile vacuole alone.

It is certain, then, that the size of the vacuole does not determine the time of systole. On the other hand, the size of the vacuole is dependent to some extent upon the time of systole, because if contraction is delayed the vacuole continues to grow larger from a constant accumulation of fluid. However, other factors besides the time of systole influence the size of the contractile vacuole because the contractile vacuole does not always grow at the same rate; that is, sometimes it reaches a certain size in a shorter interval than it does at another time even in the same animal. This increase in the collection of fluid at times must be due to slight changes in the physiological state of the protoplasm—momentary fluctuations due perhaps to an accelerated rate of oxidation forming more excretory products—or to changes in the permeability of the organism allowing more fluid to enter from the external medium.

This brings us to the question of the cause of systole. As has been shown, the stimulus does not come from within; that is, it is not caused by the pressure of a certain amount of the contained fluid because a vacuole may contract when very small. It is also quite evident that it cannot be due to any general condition of the protoplasm, because in amoebae with two contractile vacuoles the pulsations are not synchronous. Systole must be due, therefore, to some local change; and Taylor's theory, which has been described, fulfills at least this one condition. According to him, the stimulus for systole is the contact of the vacuolar wall with the ectoplasmic surface where a pore is formed due to a gel-sol reversal of these two boundaries.

The behavior of the surrounding protoplasm at systole is of importance in connection with the theory of the mechanism of systole. This behavior has been described as a movement of protoplasm away from the vacuole followed by a flow in the reverse direction. It is very probable that this latter movement, i.e., the streaming of the cytoplasm toward the vacuolar wall, indicates an increase in surface tension there with corresponding increase in adsorption of cytoplasmic substance of lesser surface tension. This movement is followed by immediate contraction. These two events indicate the extreme probability of an important rôle being played in the contraction of the vacuole by phenomena involving surface tension and including the principle of minimal surfaces. There are some grounds for the belief, then, that the mechanism of systole is primarily dependent upon changes in surface tension, which may be followed, in turn, by such sol-gel reversals in the cytoplasm as described by Taylor.

Throughout the course of the observations, both on the normal amoebae and the portions, it was evident that the usual position of the contractile vacuole was in close proximity to the nucleus. Although the contractile vacuole in Amoeba proteus is most often described as having a position posterior to the nucleus, this is by no means a constant position. It may even be separated from the nucleus for a time by quite a distance, while, on the other hand, it often overlies the nucleus. It is also described as moving to the posterior end of the amoeba before systole, but just as often it remains near the nucleus and contracts in that position. In addition, it is of interest in this connection to note that the regenerated vacuole, although newly formed probably in most cases at the periphery of the organism, invariably moves up to a position by the nucleus rather soon after its origin and even before it has attained its maximal size. This is still more striking in the few cases in which new vacuoles formed in different parts of the amoeba and then migrated to the region of the nucleus where they eventually became confluent.

It appears, then, as if there may be in the vicinity of the nucleus a physiological condition of the protoplasm which is conducive to the collection of this liquid from all parts of the organism and to the proper functioning of the vacuole, although the vacuole is in no way dependent upon the nucleus for its actual existence.

In considering the function of the contractile vacuole, the composition of the vacuolar fluid is of primary importance. Consequently, the observed reaction of the amoeba to the expelled contents of the vacuole is suggestive. The substance expelled evidently reduces the surface tension, causing the pseudopodia to project, and since CO_2 and other acids reduce surface tension, these observations suggest the acid nature of the excreta. This view of the acid nature of the vacuolar contents is further substantiated by the work of Edwards ('23), who showed that when amoebae are stimulated locally by acids small pseudopodia are projected.

The results from the experimental animals showed facts of interest from various other points of view. New contractile vacuoles were regenerated in pieces of amoeba whether nucleated or not. Although the intensive study of the contractile vacuole of enucleated portions was not a part of this investigation, it was determined that these vacuoles showed an increasingly slower rhythm of pulsation and excreted very small amounts of fluid. It is well known, of course, that an enucleated cell can no longer perform all its life processes in a normal manner, and, therefore, it is not to be expected that the contractile vacuole in an enucleate portion of amoeba would show normal behavior. Hofer ('89) observed an enucleate piece of Amoeba proteus for fourteen days which was as long as it lived. Whereas the normal pulsation of the vacuole was at seven-minute intervals, in his experiments the periods became longer and longer, and finally there was one pulsation every two hours, and even then the contents was not completely expelled. Penard ('05) considered this to be evidence of the fact that the activity of the contractile vacuole is directly proportional to the activity of the whole animal.

From many series of regeneration experiments, investigators have determined that an enucleate portion of a protozoon cannot regenerate lost parts. However, as new contractile vacuoles appear in enucleated pieces of amoeba, we must consider the contractile vacuole a different sort of structure; that is, it is not a definitive protoplasmic structure, but simply a collection of liquid in the protoplasm with a temporary membrane around it. In other words, the contractile vacuole is in no way dependent upon anabolic activity for its existence.

Another indication that the contractile vacuole is a different type of organelle is shown by the factor which determines its time of regeneration. Sokoloff ('24) came to the conclusion from a long series of regeneration experiments on various kinds of protozoa that the larger the piece is in proportion to the original organism, the sooner is the regeneration of lost parts accomplished. However, the present experiments show this is not true in the case of the regeneration of the contractile vacuole, since the time of regeneration depends upon the size of the original animal. The results obtained showed that pieces from larger amoebae regenerated a contractile vacuole sooner than those from smaller amoebae irrespective of the size of the portion itself. Since these largest amoebae were, without doubt, very near the division phase in their history, this condition must have influenced the readiness with which they were able to form a new vacuole. In the process of normal fission vacuoles are seen usually immediately after division in both daughter amoebae, each probably being a new vacuole. Before fission of the amoeba, then, the physiological state of the protoplasm is such that a new vacuole may be formed immediately, and the cutting of the animal, like normal division, gives the final stimulus, and a new vacuole is formed within a few minutes.

The new vacuoles in the nucleated portions exhibited an interesting difference in behavior according to the size of the portions. In this connection some observations of Gruber ('12) are pertinent. Carrying on physiological experiments with Amoeba proteus by the operative method, he came to the conclusion that very small pieces of amoebae have small vacuoles, but if the pieces are not much smaller than the original animal the vacuoles have almost the size of the original. Although he gave no data as a basis for this conclusion, and his observations, as far as can be told, were made on the original vacuole and not on a regenerated one, the results obtained in this paper, in general, support his statement. Furthermore, Gruber stated that the small vacuole in small portions had the same rhythm of systole as the large vacuoles of unoperated animals, and, giving data from five portions, stated his conclusion that the rhythm is not effected by operating. The present study corroborates Gruber's conclusions in regard to the small portions, as there was very slight change in the rate of pulsation; but, on the other hand, in the case of the larger portions, of which Gruber made no mention, there is a decided slowing of the rhythm.

The behavior of the regenerated vacuoles in the smaller portions as regards size and rate of contraction is the converse of that in the larger portions. The vacuoles in the small portions decrease in size, but maintain the normal rhythm, while the vacuoles in the large portions have the same size as the original, but decrease in rate of pulsation. Although the vacuoles in the small portions decrease in size considerably, the fact that the rhythm remains normal accounts for the result that these smallest portions excrete the largest quantity of fluid in proportion to their volume.

How can this comparatively large increase in the proportional amount of excretion in small portions be accounted for? If we consider the vacuole to be primarily an apparatus for the regulation of the osmotic relations of the organism, this increase in amount of fluid expelled must be explained as due to an increased intake of water by the protoplasm from the external medium. Since, with decrease of volume, the proportion of surface to volume increases, this change in relationship of surface to volume must hold in these portions of amoebae. Consequently, in a portion of amoeba with a greater proportion of surface exposed to the medium, more water may enter the organism in proportion to its volume, and, therefore, more may be excreted than is proportional to its volume. If this is true, the smaller portions of amoebae would excrete increasingly more in proportion to their volume. The results in this investigation fulfill these conditions to some extent.

On the other hand, the cause of this proportionally great activity of the vacuole in smaller portions may be attributed to an increase in the formation of excretory products caused by the stimulus of cutting. This can be considered parallel to the results obtained from operations upon planarians by Hyman ('23) and others, in which the shorter the piece, the greater was the rate of metabolism.

It is not possible to decide from the data at present in hand, however, which of these two factors is the cause of the increased excretion in smaller portions of amoebae, but it is not improbable that both injury and the change in the surface-volume relation have a share in bringing about this result.

SUMMARY

1. The contractile vacuole in an amoeba may vary considerably, within a half hour, in size and rate of contraction under apparently identical conditions.

2. As the length of time between systole increases, the size of the contractile vacuole increases; that is, the slower the rate of contraction, the larger the contractile vacuole.

3. The larger the amoeba as denoted by nuclear size, the larger is the contractile vacuole.

4. The larger the amoeba, the more liquid is excreted in a unit of time.

5. When the vacuole is cut off from an amoeba, a new one is always regenerated.

6. The larger the nucleus, the sooner the contractile vacuole is regenerated; that is, the larger animals regenerate a new vacuole more readily.

7. The rate of pulsation of the new vacuole is decreased in portions of all sizes, but very little in portions as small as one-third and one-fourth the size of the original amoeba.

8. The size of the new vacuole in the larger portions is little different from the size of the original vacuole, but in the one-third and one-fourth portions there is a significant decrease in size.

9. The volume of liquid excreted by the new vacuoles in the portions exceeds the expectation; the smaller portions, in general, excreting increasingly larger amounts in proportion to their size.

10. The process of fission was studied in Amoeba proteus, and the binucleate condition was observed to occur as a result of the failure of the completion of cytoplasmic division after nuclear division had occurred.

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REGIONAL DIFFERENCES IN RATE OF OXIDATIONS IN THE CHICK BLASTODERM AS SHOWN BY SUSCEPTIBILITY TO HYDROCYANIC ACID

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FOUR FIGURES

INTRODUCTION

In the living organism the rapidity of the oxidative reactions varies in different regions of the system. Substantial and satisfactory evidence has accumulated which shows that the effect of an agent or condition which depresses the oxidative reactions in the organism, when present in appropriate concentrations or intensities, varies with the original rate of oxidations in the system; i.e., the higher the original rate the more profound the effect produced by a depressant. The correlation between rate of oxidations and depressive effect is considerably less than unity (Buchanan, '26), hence factors other than the rapidity of oxidations are involved. It is, however, of sufficient magnitude to afford a reliable criterion of relative differences in oxidative rate within the organism.

Among the agents and conditions that are known to depress biological oxidations, hydrocyanic acid is of the most powerful, even when applied in dilute concentrations. An attempt is made in this paper to detect regional differences in rate of oxidations in the chick blastoderm by the analysis of the effects of HNC on the system.

MATERIAL AND METHODS

The eggs used in these experiments were obtained the day after laying and subjected to experimental conditions shortly after being received. The apparatus used and the methods of procedure were simple. A glass jar, capacity about 3 liters, with a wide mouth engaging a ground-glass lid, was used as the egg container. The lid was bored to receive a rubber stopper, the stopper being bored to receive inlet and outlet glass tubes. The eggs, four to six at a time, were placed in the container and the lid sealed with paraffin. The outlet tube was connected with a filter pump attached to a faucet. The inlet tube was connected by a rubber tube 9 ft. long with the outlet tube of a flask containing the desired concentration of KNC. The inlet tube of the flask opened to the air of the room and dipped to the bottom of the KNC solution in the flask. The egg container with its long rubber inlet tube was placed in the incubator, the flask containing the KNC solution being placed on a table near by. After the incubator had been properly closed, the water at the faucet was turned on, and a slow current of air was thus drawn first through the cyanide solution, then through the coiled rubber inlet tube where it was warmed, and finally through the egg container. A thermometer was placed in the egg container and observed from time to time to assure that the temperature within the jar was approximately the same as that of the incubator. It was found in preliminary experiments that air thus drawn through a KNC solution contained sufficient HNC to affect the development of the chick. No attempt was made to determine the proportion of HNC in the air current, since this would vary with the carbon-dioxide concentration of the air drawn through the KNC solution, with variations in temperature of the incoming air, with the rapidity of the air current, and with other conditions not readily controlled. The concentration of HNC was roughly regulated by increasing or decreasing the concentration of the salt in solution. During the course of incubation the solution of cyanide was replaced by fresh at intervals of about six hours, except at night, when the same solution was used continuously for periods up to twelve hours.

Under these conditions, the HNC reaches the blastoderm in extremely dilute solution in the egg-white.[1] From time to time control experiments were carried out in which groups of six eggs were sealed in the container, and the air current bubbled through water instead of KNC was drawn through. Careful examination of thirty-six chicks incubated in this manner failed to discover any defects or retardation of development.

After the desired period of incubation, the eggs were removed and opened under physiological salt solution, the blastoderms dissected off and preserved in Bouin's or other appropriate fixative, then cleared, stained, and mounted in the usual way. The period of incubation was limited in all experiments to ninety-six hours or less.

RESULTS

While the effect of the HNC varies in different lots of eggs, in general the intensity of effect depends upon the concentration of KNC employed as the source of HNC. In all, 120 fertile eggs were treated, of which ninety-one show distinct defects in development. The experiments may be divided into three groups: 1) continuous incubation in HNC, designed to test its general and local effects; 2) incubation in HNC followed by incubation in air, designed to test the recovery powers after treatment with HNC; 3) incubation in air, then in HNC, followed by a second period of incubation in air, designed to test, *a*) the periods of development during which susceptibility is greatest and, *b*) recovery processes, as in group 2.

The general nature of the defects appearing in all three groups will be described together. Since slight variations in stage of development and form are to be expected in the early periods of incubation, only distinct and recurring abnormalities are here regarded as due to the effects of the treatment. As criteria of the effects, normality of body form, of the neural tube, and of the circulatory system have been used.

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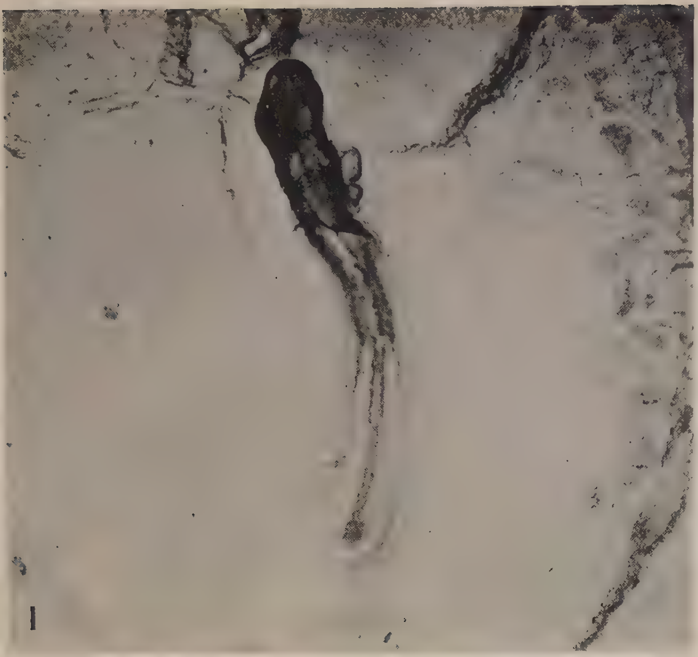
[1] No data on the solubility of HNC in egg-white are available, but at normal pressure and incubator temperature it may be regarded as less than in water.

BODY FORM

Abnormalities of body form produced by exposure to HNC range from slight abnormality of the configuration of the anterior end and exaggeration of the dorsal flexure to almost total suppression of development. In general, the entire blastoderms are distinctly less extensive than that of a normal chick of equal incubation. Affected blastoderms are usually conical in shape, indicating a greater restriction of the advance of the marginal periblast than of the expansion of the central region of the blastoderm. Affected embryos show all degrees of inhibition, but the greatest effects are evident in the anterior region (fig. 1). In many cases the relatively abrupt inhibition of the head without corresponding effect on more posterior regions causes the body of the embryo to be so impacted and thrown into a confusion of folds that analysis is profitless. Even in relatively mild cases the dorsal flexure is much exaggerated—a defect similar in appearance to one frequently found in incubating chicks (fig. 2). In every case, however, the number of somites is less than normal. In two cases, to be mentioned later, the inhibition of the head is so complete that the heart is located in front of the head fold. In cases of extreme inhibition of the development of the head, the head fold of the amnion is also suppressed. In other affected embryos the amnion is more or less inhibited.

Table 1 gives a summary of the number of defective embryos derived from continuous exposure to a stream of air containing HNC. In this and in following tables distinct defects are listed as 'minor,' and major malformations as 'major.' Examination of all embryos affected shows that the regions of greatest susceptibility to HNC when applied at the beginning and throughout incubation are the anterior end of the embryo and the margin of the blastoderm. In experiments with stronger HNC, the body of the embryo is profoundly inhibited and in some cases entirely absent, its site on the blastoderm being indicated by a space completely devoid of cells, although the area vitellina was present in all



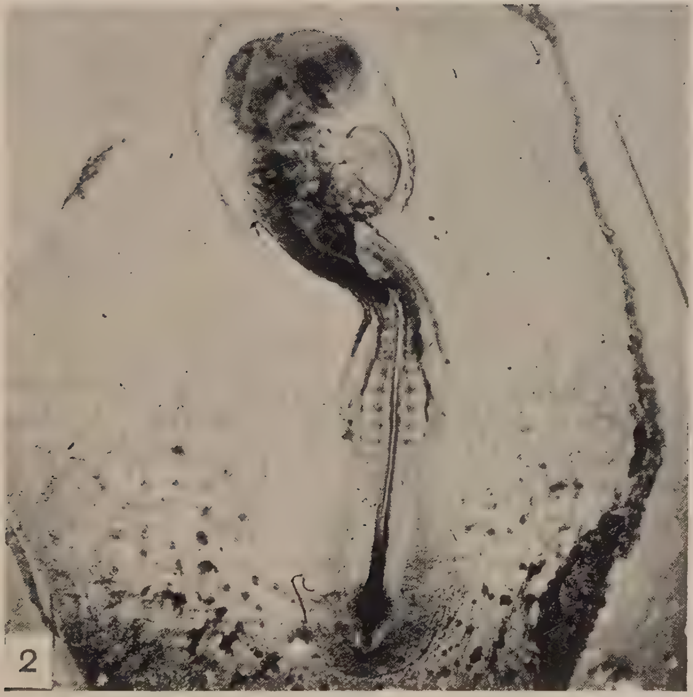


Fig. 1 Chick incubated fifty-two hours in a continuous stream of air drawn through M/125 KNC. × 15.

Fig. 2 Chick incubated fifty-two hours in a continuous stream of air drawn through M/500 KNC. × 15.

cases. The effect of the HNC is therefore both general and local. The resulting blastoderms show a general retardation of development. Moreover, greater inhibitory effects are evident in the embryo than in the extra-embryonic blastoderm. The effect on the margin of the blastoderm is greater than in the central region, and the anterior end of the embryo is more inhibited than regions more posterior.

NEURAL TUBE

Abnormalities of the neural tube are very common, showing that the embryonic nervous tissue is relatively highly susceptible to cyanide depression. The suppression of the forebrain is most frequent and, concurrent with this abnormality, is the formation of convolutions along the remainder of the neural tube, due in part to the fact that regions posterior to the brain are appreciably less inhibited than the anterior end and in part to the mechanical interference with its elongation (fig. 1). There is frequent failure of the neural tube to close in its normal manner. In relatively severe cases not only is the forebrain inhibited, but also the mid- and hindbrain show greater depression than regions more posterior. The optic vesicles do not appear to be particularly susceptible, defects occurring only in conjunction with other and greater abnormalities. The regions of greatest susceptibility in the nervous system are, in order: the forebrain, the crests of the neural folds, and the mid- and hindbrain.

TABLE 1

Continuous exposure to dilute HCN

NUMBER OF FERTILIZED EGGS	CONCENTRATION OF KCN	HOURS OF INCUBATION	DEFECTS		
			Major	Minor	Total affected
6	M/500	28	6	0	6
2	M/1000	52	2	0	2
6	M/1000	52	6	0	6
5	M/125	52	5	0	5
6	M/125	24	6	0	6
Totals 25			25		25

CIRCULATORY SYSTEM

The abnormalities appearing in the embryonic circulatory system after exposure to HNC are of two general types: 1) abnormalities of the heart and, 2) the accumulation of blood in large sinuses near the site of normal entry of the omphalomesenteric arteries into the area vasculosa. Stockard ('14) also found this latter condition of frequent occurrence in eggs treated with certain anesthetics before incubation.

Abnormalities of the heart are chiefly to be described as abnormalities in relative size of the venous to the arterial regions, shortening of the anteroposterior dimension, and greater flexion (figs. 1 to 3). In two cases, noted above (p. 144), the head of the embryo was extremely inhibited, apparently prior to the time of approximation of the amniocardiac vesicles, so that these were located anterior to the masses of head tissue. They (the endocardia) had failed to combine, and when the eggs were opened the two vessels were beating independently and asynchronously. A striking fact in these experiments was the paucity of oxyhaemoglobin in the blood. In some embryos the blood was to all appearance free from red coloring, while in many others the color was distinctly subnormal. Stockard ('14) noted this condition in chicks incubated in the presence of anesthetics and Riddle ('21) in dove embryos incubated in deficient oxygen.

RECOVERY

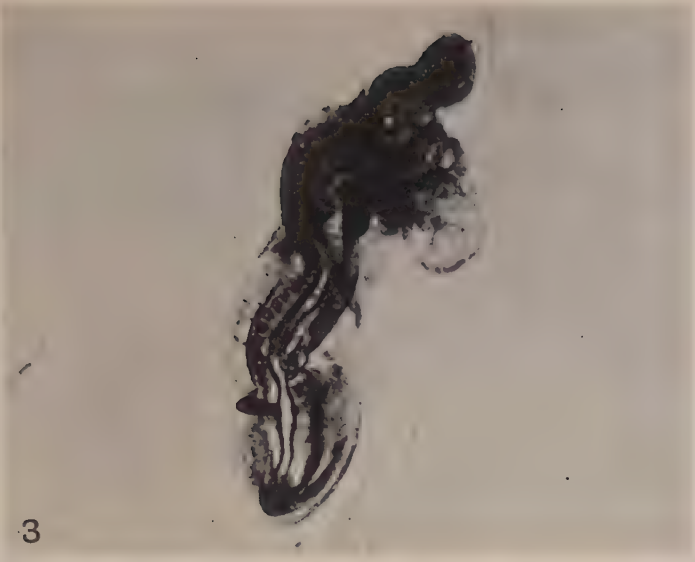
The ability of the embryo to recover from the effects of HNC was tested in the following manner: Eggs were incubated as in experiments with a continuous stream of air containing HNC, and, after a certain period, the cyanide flask was disconnected, unpoisoned air being thus admitted. The eggs were then opened after various periods of exposure and recovery. The treatment with HNC ranged from the first sixteen hours to thirty-six hours of incubation, the total incubation ranging from forty-eight to seventy-two hours. In all, thirty-four eggs were thus treated. Table 2 gives a summary of the number and extent of defects observed.

The abnormalities consisted of further complications of those already described. In experiments in which the HNC was applied during the early periods of incubation the most frequent effect observed was the failure of the anterior end of the embryo to recover (fig. 3). The trunk region was frequently thrown into folds and more or less complicated masses, being distorted in some individuals and in others split into well-separated halves. Presumably, the distortion of the midregion was in large measure due to a less profound effect of the HNC and a more complete recovery, while the anterior end, being more inhibited, prevented elongation. In extreme cases the entire embryo showed little evidence of recovery. In two cases the neural tube gave off branches laterally (figs. 3, 4). This fact is worthy of further comment, indicating the profound effect of the HNC not only as a depressant of the processes of development of the nervous system, but also that this depression may be accompanied by a partial obliteration of the normal anteroposterior axiation to such a degree that the embryonic nervous tissue may, on recovery, develop new partial axes. It may further be noted that several of these axes are at approximate right angles to the long axis of the neural tube, i.e., the branching is not always dichotomous. The new axes appear to have no relation to proximity to a circulatory channel. Obliteration of

TABLE 2

Recovery from depression by dilute HCN

NUMBER OF FERTILIZED EGGS	CONCENTRATION OF KCN	HOURS OF INCUBATION IN HCN	HOURS IN AIR	TOTAL INCUBATION	DEFECTS		
					Major	Minor	Total
6	M/125	36	24	60	6	0	6
4	M/150	24	48	72	4	0	4
1	M/150	27	45	72	1	0	1
6	M/250	24	48	72	1	5	6
6	M/200	23	49	72	2	3	5
6	M/250	16	32	48	0	0	0
Totals 29					14	8	22



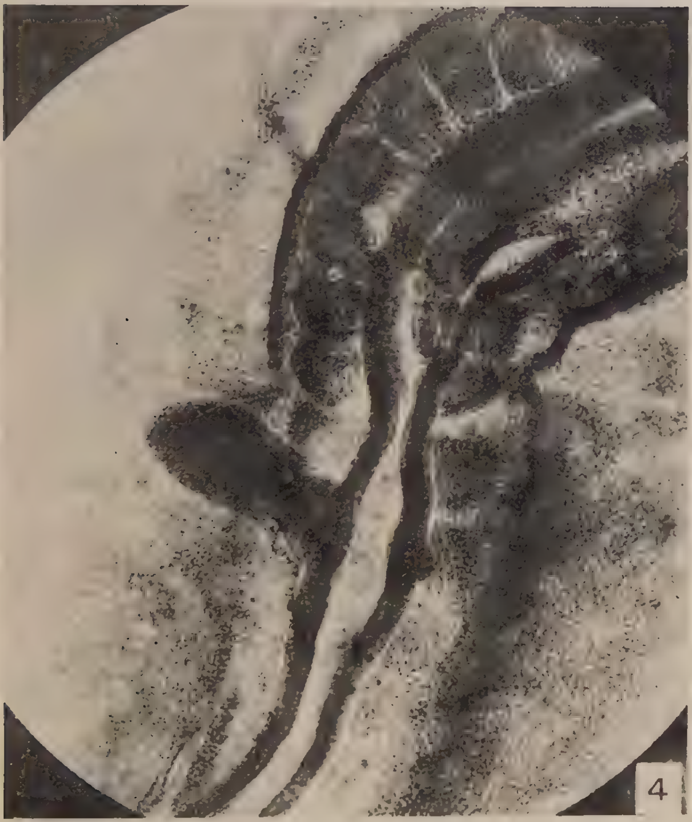


Fig. 3 Chick incubated twenty-four hours in a stream of air drawn through M/200 KNC, followed by forty-eight hours' incubation in air. Total incubation, seventy-two hours. × 15.

Fig. 4 An enlargement, × 75, of the posterior region of the chick shown in figure 3.

the physiological polarity of many organisms has been accomplished by a number of depression methods. The chick embryos with branched neural tubes show that similar methods may partially obliterate the physiological polarity of the embryonic vertebrate nervous system.

STAGE OF SUSCEPTIBILITY

From the foregoing it is evident that the developing chick embryo is profoundly affected by exposure to minute quantities of HNC, the effects being, in general, different only in degree and depending largely on the concentration of HNC, the duration of exposure, and whether or not recovery has occurred. The appearance of the material indicates that the anomalies are true inhibitions of development, and not degenerative changes. The question as to the stage of development which is most sensitive to HNC presents itself.

A series of experiments was carried out in which the eggs were placed in the container as before, and the air current, instead of being drawn through a solution of KNC, was drawn through tap-water for a certain period, and then for a later through the KCN solution. In a second group in this series the treatment with HNC was followed by a second period of incubation in air. Table 3 gives the results. The defects observed varied in degree, but are chiefly to be attributed to retarded development of the head. In those embryos in which the treatment with HNC followed a period of incubation in air (approximately one-half day), the effect of the HNC was distinctly less marked than in previous experiments, particularly in regions posterior to the head. Certain minor abnormalities of the circulatory system appeared, the ventricular region of the heart being somewhat exaggerated in some individuals, and in some abnormal sinuses at the level of the omphalomesenteric arteries were formed. It appears, therefore, that the susceptibility of the embryo decreases during the early period of incubation, but the susceptibility of the head and certain other structures remains higher than that of the embryo exclusive of these parts; i.e., there is a decrease is the absolute susceptibility of the embryo, but a less marked change in the relative susceptibility of its parts.

GENERAL CONSIDERATIONS

A substantial body of evidence has accumulated which shows that in the development of the embryo of both vertebrates and invertebrates, as well as in many adult forms of the more simple organisms, certain regions act as controlling elements, influencing and determining in some way the developmental fate and initiating and controlling the early differentiation of other regions more or less distant. Recently Spemann ('18 and later papers) has demonstrated in a convincing manner the very great importance of 'organizators' in vertebrate development.

TABLE 3

Stage of susceptibility. Recovery from HCN depression

NUMBER OF FERTILIZED EGGS	CONCENTRATION OF KCN	INCUBATION				DEFECTS		
		Hours in air	In HCN	In air	Total	Major	Minor	Total
1	M/1000	7	16	5	28	1	0	1
1	M/1000	7	16	29	52		1	1
4	M/250	7	17	28	52	4	0	4
4	M/250	7	17	28	52	0	0	0
4	M/250	6	24	22	52	1	1	2
6	M/250	16	24	24	64	0	0	0
6	M/125	24	12	14	52	2	4	6
6	M/125	7	34	19	60	6	0	6
4	M/250	8	15	49	72	4	0	4
6	M/125	8	16	48	72	6	0	6
6	M/125	17	9	46	72	3	3	6
6	M/125	18	12	66	96	6	0	6
6	M/1000	28	24		52	0	2	2
6	M/500	12	17		29	0	6	6
Totals 66						33	17	50

Extended studies of the physiological conditions obtaining in these dominant regions shows that they are characterized by relatively higher rates of metabolic activity than more subordinate regions. This fact has been clearly demonstrated in the adults of more simple organisms by the higher oxygen requirement of dominant regions, their more rapid carbon-dioxide output, greater susceptibility to poisons, and other criteria. According to Child ('15 and later papers), dominant regions exercise control over subordinate regions by reason of their greater metabolic activity. A difference in electrical potential between dominant and subordinate regions has been demonstrated in many cases (Hyman and Bellamy, '22), and the suggestion has been advanced that the attendant bioelectric phenomona may be the effective factor in this correlation (Child, '21; R. S. Lillie, '23).

According to this conception of correlation in development, the problem of teratogenesis is indissociable from the problem of the pattern or distribution of regions of higher and lower rate of metabolism in the egg and embryo and of changes during development. Stockard ('21, pp. 220, 223) has stated the problem in somewhat similar terms, but does not regard such differences in rate of metabolism as having been demonstrated. He is also unable to account for the origin of quantitative differences of this sort. Since the origin of differences in rate of physiological processes constitutes one of the chief problems of development, Stockard's difficulty is one common to all who are interested in the problem. It may be pointed out, however, that in agamic reproduction it is known that in many cases the differences in rate of metabolism within the new individual represent the orientation relation to the parent organism. It is also known that in some cases a number of environmental conditions that are effective in disturbing the rate of metabolism differentially where differences already exist are responsible for the origin of such differences. While it is true, as Stockard points out, that the nature of the mechanism of control in correlation between regions of high and low rate of metabolic activity is not known, the fact that such correlations represent a type of protoplasmic transmission has been demonstrated. To describe protoplasmic transmission as a 'problematical factor' seems undesirable.

In the interpretation of development adopted here, the terms 'arrest of development,' 'developmental rate' (Stockard, '21) and 'cephalocaudal differential growth' (Kingsbury, '24) may be employed only as descriptions of morphological facts, the end products of certain of the physiological processes of the embryo. These facts have long been known, but of themselves have yielded no advance in the knowledge of the physiology of development, nor any information as to the nature of the mechanism that is responsible for their origin and maintenance.

We have, then, to consider the problem of the nature of the action of the cyanides on protoplasm. The known biological effects of the cyanides include, *a*) arrest of viscosity changes (Heilbrunn, '20); *b*) changes in permeability to a wide range of substances (Krehan, '14; Niina, '24); *c*) profound depression of metabolic activity, particularly oxidative metabolism (Hyman, '19; Buchanan, '26). Relatively stronger concentrations of the cyanides have been employed in detecting changes in viscosity and permeability, while extremely dilute concentrations are powerful depressants of oxidative metabolism. Since in these experiments the HNC was applied in extremely dilute concentrations, osmotic effects, effects on viscosity, on acidity, and on permeability, while perhaps involved in the depression produced may be regarded as minor rather than primary causes of the developmental defects observed. It seems reasonable to conclude that the depressive effects of HNC on the development of the chick embryo are a consequence of its depressing action on protoplasmic oxidations.

As to the nature of the action of the cyanides in interfering with protoplasmic oxidations, the evidence is not decisive. Whatever the mechanism of its action may be, however, it has been shown that the severity of its effect in depressing biological oxidations depends to a marked extent on the original rate of oxidative activity, i.e., the higher the original rate, the more profound the depression (Buchanan, '26; Galigher, '21).

The results of the effect of HNC on the development of the chick embryo may be said to indicate that the regions in which the inhibition of development is greatest are regions of greatest oxidative activity in normal development. If this conclusion is correct, these results may be interpreted as follows: 1) The development of the early blastoderm of the chick involves oxidative processes that are highly susceptible to depression by HNC. 2) The oxidative activity of the margin of the blastoderm (presumably the marginal periblast) is more rapid than that of the central region. 3) Oxidative activity is greater during the early hours of incubation than later. 4) Oxidative activity is greater in the embryo than in the extra-embryonic blastoderm. 5) Within the embryo oxidative activity is greatest in the anterior end. 6) The closure of the neural tube is associated with relatively high oxidative activity in the crests of the neural folds. 7) Fusion of the amniocardiac vesicles also involves relatively high oxidative activity. 8) The normal form of the heart depends, to some extent at least, on differences in oxidative activity in its several regions. 9) A very considerable number of mechanical factors concerned in the development of normal form are associated with, and depend on, regional differences in rate of oxidations. The evidence also indicates that recovery of a region from depression of oxidative metabolism depends to a marked extent upon the degree of depression and consequently upon the original rate of oxidations.

Bellamy ('19, '22) has described the regions of greatest physiological activity in the frog embryo, and Hyman ('21) and Hinrichs ('25) have made similar studies of the teleost embryo. The analysis of the loci of regions of greatest physiological activity in the developing chick has not yet been accomplished by direct methods. Shearer ('24) has attempted to measure the oxygen consumption of various regions along the long axis, and states that the anterior end is highest in rate of oxygen consumption, the rate decreasing posteriorly. While there is a general agreement between his results and the fact, reported here, that the anterior end is most susceptible to HNC, as Hyman points out ('25), Shearer has simply demonstrated that the head, containing an excess of metabolically active nervous tissue, consumes more oxygen than regions which contain proportionately less.

Excepting the cases of the rise of new axes in the neural tube, the results reported here contain no clear evidence that regions of relatively low physiological activity undergo development more rapidly when regions of higher activity are inhibited. All that is shown is the fact that certain regions are more inhibited than others. There is also no evidence of any shifting of the site of origin of parts or organs, except such dislocations as may reasonably be ascribed to mechanical factors. Except in the cases of permanent separation of the amniocardiac vesicles resulting in two abnormally shaped hearts, there is also no evidence of reduplication of parts. The reasons for this apparent lack of evidence of dominance and subordination in the development of the chick are probably to be found in the conditions existing in the chick's egg at the same time of incubation and in the nature of the experimental method. Before it is laid, the egg undergoes an important period of development. Gastrulation occurs, and the thickening of the ectoderm in the center of the area pellucida already indicates the primordium of the nervous system. When incubated in HNC, time is required for the depressant to reach the blastoderm. While the shell and membranes probably do not offer any serious obstacle to penetration of the HNC, the egg-white in all probability does. It is likely, therefore, that, due to the fact that the HNC must diffuse through the egg-white, the cyanide is not present in sufficient concentration to affect oxidative metabolism materially until some time after the beginning of incubation. Hasselbalch ('02) showed that the hen's egg gives off oxygen during the early period of incubation, which is explained by Krogh ('16, p. 109) as due to the giving off at incubator temperature of oxygen dissolved in the egg substance at a lower temperature. An adequate supply of oxygen is thus present in the immediate environment of the blastoderm at the time incubation starts, but some time must elapse before the HNC has diffused through the egg-white and has reached the developing embryo.

It is therefore reasonable to conclude that the embryo develops well beyond its condition at the time of laying before the HNC is present in sufficient concentration to inhibit development. By this time differentiation has proceeded and many structures are probably established as self-differentiating systems (Hoadley, '24 and later papers). The early appearance of a circulatory system presumably sets in operation a whole system of correlations effected by transported substances. The rôle of differences in quantitative physiological condition as factors in controlling differentiation may be regarded as becoming increasingly less important as differentiation becomes more and more complete. Hence the application of HNC to a system already largely composed of self-differentiating parts and soon correlated by a common circulatory medium may not reasonably be expected to alter the sites of origin of structures and organs, nor to divide primordia already differentiated so that reduplication of parts occurs in subsequent development.

SUMMARY

An analysis of the effects of hydrocyanic acid on the development of the chick up to ninety-six hours of incubation indicates: 1) The development of the early blastoderm of the chick involves differential oxidative activity in its several regions. 2) The rate of oxidations of the margin of the blastoderm is higher than that of more central regions, excepting the embryo. 3) The rate of oxidations is higher during the early hours of incubation than later. 4) Oxidative activity is more rapid in the embryo than in the extra-embryonic blastoderm. 5) Within the embryo the regions of greatest oxidative activity are the anterior end, including the brain, the crests of the neural folds, and the sinus region of the heart. 6) Differential depression of oxidative metabolism results in interference with mechanical factors in normal form development.

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STUDIES ON AMPHIBIAN METAMORPHOSIS

I. FORMATION OF THE OPERCULAR LEG PERFORATION IN ANURAN LARVAE DURING METAMORPHOSIS

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TWO TEXT FIGURES AND SIX PLATES (TWENTY-EIGHT FIGURES)

CONTENTS

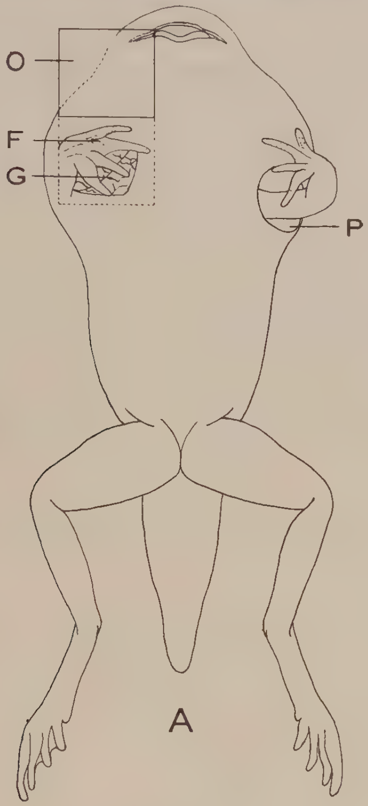
Introduction 2
Materials and methods 6
Formation of a normal opercular perforation 8
  1. Macroscopic sequence of events 8
  2. Histological sequence of events 10
  3. Enlargement of the perforation 11
  4. Union of the various layers of the opercular and fore-limb integuments 12
Specificity of the integument 13
  1. Purpose of the skin transplantations 13
  2. Technique of skin transplantation 13
  3. The healing process 14
  4. Macroscopic changes in appearance of grafts 15
Influence of the fore limb 19
  A. Extirpation of the right fore limb 19
    1. Purpose of the experiment 19
    2. Technique of limb extirpation 20
    3. Extirpation of fore limb of R. pipiens 20
    4. Extirpation of fore limb of R. sylvatica 22
    5. Extirpation of fore limb of R. clamitans 24
  B. Fore-limb transplantations 25
    1. Purpose of the experiment 25
    2. Technique of homoplastic fore limb-bud transplantations 26
    3. Homoplastic limb-bud transplantation in R. palustris 26
  C. Glass-bead implantations 29
    1. Purpose of the experiment 29
    2. Technique of glass-bead implantations 30
    3. Bead implantation in R. clamitans and R. pipiens 30
Histolytic function of atrophying gills 33
  1. Purpose of the experiment 33

2. Technique of gill-transplantation experiments 34
3. Homoplastic gill and skin transplantation in R. palustris 35
4. Homoplastic gill transplantation in R. palustris 37
5. Homoplastic gill and skin transplantation in R. pipiens 39
Discussion 41
General summary and conclusions 51
Literature cited 54

INTRODUCTION

The problem with which we are particularly concerned in the present paper has to do with the factors involved in the formation of the opercular perforations through which the developing fore legs are released during metamorphosis. This immediately raises the question whether or not the thyroid hormone brings about an histolysis of the opercular integument or whether it acts indirectly through a chain of intermediate factors. It is a well-known fact that the fore legs develop in the opercular cavity. The operculum bounding this cavity first arises as a fold of skin on either side of the head at a time when the external gills of the larvae are replaced. This fold gradually extends backward, the free posterior edge of which finally fuses with the body behind and below the gill region. The opercular cavity, so formed, is totally enclosed, therefore, by an integumentary layer with the exception of a small space on the left side, the spiracle, which serves as an outlet for the water supply of the internal gills.

During the metamorphosis of the larvae, the fore limbs, previously represented only by small buds, differentiate very rapidly and soon attain considerable proportions. They are typically bent upon themselves at the elbow (*F*, text figure A) and occupy a large portion of the opercular cavity. The internal gills, which are in a state of atrophy at this time, are generally seen lying directly beneath the hand of the fore limb (*G*, text figure A) or in the immediate vicinity of the latter. At a rather late stage of metamorphosis, the imprisoned fore legs are finally released through well-defined holes or 'perforations' (*P*, text figure A) which appear in the operculum of that region. According to Barfurth ('94), the right fore leg is generally released first, the left following shortly afterward. Evidently, Barfurth meant to say that the fore leg on the right, as viewed from the ventral surface, is first released. Since Barfurth's observations were made, numerous workers have noticed the tendency of the left fore limb to appear shortly before the right fore limb. The writer has found this to be true in the large majority of cases in the larvae of the five species of frogs under investigation. Braus ('06) also states that such is the case during the metamorphosis of Bombinator larvae.



Text figure A

The first work on the particular problem with which we are concerned was that of Braus ('06). Braus extirpated the fore legs of Bombinator larvae during all stages of their development, from the bud stage up, and found that in all cases perforations still occurred in the operculum when the tadpole metamorphosed. These he found, however, were much reduced in size as compared with those formed in the presence of fore limbs. He concluded, therefore, that the fore legs are not essential to the formation of opercular perforations and that other factors must be involved. Schulze ('24) has recently repeated Braus's experiments, using larvae of R. esculenta, and likewise finds perforations forming during metamorphosis in the complete absence of the fore limbs. Both investigators, while offering no definite solution of the problem, were inclined to believe that opercular skin is a self-differentiating structure which is able of itself to form perforations during a certain stage of metamorphosis.

The general methods of attack followed in the present investigation were briefly as follows: The fore limbs of several species of tadpoles were extirpated to see whether or not perforations would form during the metamorphosis of the larvae. Skin transplantations were next carried out, which involved the transplanting of opercular integument to other regions of the body and the transplanting of skin from the back and side to the opercular region. The question of a possible self-differentiating system being present in the opercular integument was thus investigated. Fore limbs were also transplanted to the back to determine their relationship, if any, to the formation of normal[1] opercular perforations. The possible effects of pressure exerted by the fore limbs against the opercular integument were determined by the implantation of glass beads beneath the integument of the side. Finally, gill transplantations were employed, the gills being transplanted while in various stages of atrophy, beneath the integument of the back or beneath opercular skin grafts placed on the back. The following series of experiments, which will be described in detail under their respective sections, were performed:

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[1] 'Normal opercular perforation' refers to the perforations which occur in the operculum of tadpoles undergoing a natural metamorphosis and through which the developing fore legs are released.

1. Autoplastic skin transplantations (fifty cases): R. clamitans. Autoplastic, opercular, skin grafts from right side interchanged either with side or back skin grafts.
2. Fore-limb extirpations (eighteen cases): R. clamitans. Right-side fore limbs extirpated.
3. Fore-limb extirpations (thirty-four cases): R. pipiens. Right-side fore limbs extirpated.
4. Fore-limb extirpations (fifteen cases): R. sylvatica. Right-side fore limbs extirpated.
5. Homoplastic limb-bud transplantations (eighty-four cases): R. palustris. Homoplastic right fore-limb-bud transplants into muscle of the back.
6. Homoplastic fore-limb transplantations (ten cases): R. clamitans. Right fore limbs (differentiated) transplanted homoplastically under back skin.
7. Bead implantations (twenty-four cases): R. clamitans and R. pipiens. Glass beads implanted under skin of right side just anterior to hind limb.
8. Homoplastic gill and skin transplantations (forty-eight cases): R. palustris. Homoplastic gill and opercular right-side skin grafts to back of tadpole.
9. Homoplastic gill transplantations (fifteen cases): R. palustris. Homoplastic gill transplants under back skin of tadpole.
10. Homoplastic gill and skin transplantations (twelve cases): R. pipiens. Homoplastic gill and opercular right-side skin grafts to back of tadpole.
11. Homoplastic muscle and skin transplantations (fifteen cases): R. palustris. Homoplastic tail muscle and opercular right-side skin grafts to back of tadpole.
12. Homoplastic skin transplantations (sixteen cases): R. palustris. Homoplastic opercular right-side skin grafts to back of tadpole. A control series.

This investigation was undertaken at the suggestion of Dr. W. W. Swingle, to whom the writer wishes to express his grateful acknowledgments for his helpful suggestions and kindly interest, at all times, during the course of the work. The writer is also indebted for the facilities tendered him by the Biological Laboratory, Cold Spring Harbor, Long Island.

MATERIALS AND METHODS

The larvae of five species of frogs were used in the experiments, viz., R. clamitans, R. pipiens, R. palustris, R. sylvatica, and R. catesbeiana. The last-named species was found to be unsuitable, due to the fact that it is extremely difficult to metamorphose artificially. The various stocks used were obtained from three sources: from ponds in the immediate vicinity of New Haven, Connecticut; Seymour, Connecticut, and Cold Spring Harbor, Long Island. The tadpoles were collected, for the most part, in the summer, although some were obtained in the spring, and a small number of the larvae of R. catesbeiana during the winter.

In general, only the larger individuals were used for experimental purposes, since older stages were found to lend themselves more favorably to the experiments in hand, due to the fact that precocious metamorphosis could be more easily and fully induced artificially than in the case of younger animals. The exact measurements of the individuals used will be given in the proper place.

The tadpoles, when brought to the laboratory, were generally fed for several days on Spirogyra, preparatory to operating. Tadpoles, so fed, invariably withstood the shock of the operation and the subsequent strain of artificially induced metamorphosis much better than those fed otherwise or not at all. Furthermore, it was found advisable to let the stock remain under laboratory conditions for five or six days before selecting those to be used in the experiments. During this period most of those that were unable to adapt themselves to the new environment died, thereby leaving only the more hardy members from which the desired stages were selected. Operated animals were kept in individual small battery jars.

For inducing precocious metamorphosis many of the well-known artificial methods, such as feeding desiccated thyroid, amphibian-gland transplants, and extract injections, etc., were tested, but immersion in a solution of di-iodotyrosin was found to give the most satisfactory results. Di-iodotyrosin, although a distinctly different substance as compared with the thyroid hormone, is nevertheless thyroid-like in its ability to induce metamorphosis. Because of this fact, it has been employed by a number of workers in bringing about precocious metamorphosis. It also has several distinct advantages over such methods as the feeding of desiccated thyroid (Helff, '23). Di-iodotyrosin is not a commercial product and consequently was prepared by the author according to the directions of Wheeler and Jamieson ('10).

With the exception of R. palustris, it was found necessary to anaesthetize all animals used, before making any one of the five types of operations. The animals were anaesthetized in a 0.05 per cent aqueous solution of chloretone (trichlor-tertiarybutyl alcohol; Parke, Davis & Co.) for from three to ten minutes, depending on the species and type of operation. In general, anaesthetization was continued for about thirty seconds after the animal had failed to respond to mechanical stimulation. A considerable variation was found to exist in regard to the anaesthetization period necessary for animals of the same species and of the same size and stage of metamorphic development. Frequently it was necessary to prolong the anaesthetization period.

Following the operation, the animals were placed in shallow water, so that only the wound area or areas were exposed to the air. Skin grafts were found to adhere after a half-hour's exposure to the air, while extirpation cuts and the like required an hour or more exposure to insure sufficient adhesion of the cut edges of integument. The period of anaesthetization was so regulated that the animals recovered shortly after the close of the adhesion period, at which time they were put into their individual aquaria for observation.

FORMATION OF A NORMAL OPERCULAR PERFORATION

1. Macroscopic sequence of events

The first visible external evidence of the formation of the opercular perforation is found in the coloration of the skin which is undergoing histolysis. The skin in this area assumes a lighter shade, barely noticeable at first, but in time, as the histolysis progresses, all pigmentation may be lost so that the area appears white or yellowish in color. This is due, partially, to an actual thinning of the skin and also to a contraction of the melanophores. In some cases this area of histolysis is definitely outlined by a thin white line which appears before any other noticeable change, either macroscopic or histological, has occurred in the skin so enclosed. In such cases the enclosed area always undergoes histolysis, although the extent of the perforation may be much greater. Such 'outlining' is quite frequent in R. catesbeiana and R. clamitans and rather infrequent in R. pipiens, R. sylvatica, and R. palustris.

Perhaps the most prominent external feature is that of a distinct bulging of the opercular skin, due to the pressure of the rapidly growing fore limb within the opercular cavity. This bulging cannot be placed at any one definite point in the sequence of events, since it may occur at practically every phase. Thus, in R. catesbeiana and R. clamitans, it generally precedes the histolysis, and, in fact, the opercular skin may be bulged out under considerable pressure for a week or more before any signs of histolysis are detectable. In other cases bulging may never occur, due to the rapidity of histolysis in comparison with the growth of the fore limb. It not infrequently happens, therefore, that large perforations are formed before the fore limbs have developed to any great extent. This is generally the rule in the metamorphosis of small tadpoles, especially if precocious development has been induced by means of artificial agents.

Where bulging does precede perforation, the latter generally begins as a minute hole situated at the point of greatest pressure, i.e., directly over the elbow. Generally, within a few hours, the elbow may be seen protruding through this small opening, and if the pressure is great enough, the skin immediately surrounding the hole is ruptured and the entire fore limb released. In other cases, however, a considerable area may histolyze completely before the limb is released. In either case, the initial minute perforation steadily enlarges until it reaches considerable proportions. The extent of the perforations on either side of the body may be so great that a union is almost established in the midventral line. This is more apt to be true of individuals undergoing rapid metamorphosis and especially so of younger tadpoles. It is interesting to note here, however, that the edge of the perforation is always regular in outline regardless of its size or the rapidity with which it has developed.

The fore limb has acquired its own integument some time before its release from the opercular cavity. Furthermore, this integument has established connections with the skin of the side some distance caudad to the posterior edge of the future perforation. Consequently, when the perforation occurs and the limb is released, the skin posterior to the perforation resembles a 'shelf,' which is protruding over the integument arising from the fore limb. This condition is clearly indicated in figure 6. Figures 7, 8, and 9 picture the fate of this 'shelf' of skin. It undergoes a gradual atrophy, and in time is entirely lost. The result is a complete smoothing out of the skin in that area. The last point to smooth out is at the anterior-ventral edge of the perforation. Here the process is not completed in some cases for a week or more after the tadpole has practically reached the end of metamorphosis. At this point the fore limb abuts against the edge of the perforation so that the latter is brought strongly in contact with the integument of the former. The atrophy of the 'shelf' at this point results in the complete disappearance of the perforation. The outline of the perforation is visible for some time afterward, however, as a distinct line marking the fusion points of the two integuments.

This line of demarcation becomes fainter and finally disappears as the frog develops.

2. Histological sequence of events

Sections through the opercular skin in the perforation area, made before and during the thinning process, show that the various layers undergo a process of histolysis which progresses in a very definite and orderly manner. The first noticeable histological change to occur is found in the appearance of the stratum-compactum layer. Several days before any external signs of thinning appear, the fibrils of this layer are thrown into much more pronounced convolutions than they ordinarily assume (*X*, fig. 1). This seems to be due to a crowding together of the layer or a compression of the fibrils at this point, since nuclei are more numerous in this region than elsewhere. Shortly afterward, the fibrils begin to dissociate from one another and the normal wavy appearance of the layer is consequently lost. At about this time there is an invasion of blood cells, chiefly lymphocytes, which make their way in between the fibrils of the stratum compactum and seem to function as phagocytes (fig. 2).

The stratum spongiosum next undergoes a rapid disintegration and is likewise invaded by lymphocytes, which can be found at this stage in practically all layers of the dermis and particularly in the subcutaneous connective tissue beneath, apparently migrating toward the area of histolysis. Up to this stage there is no histological sign of an actual decrease in thickness of the skin, and this probably accounts in no small measure for the absence of external signs of the process. The appearance of thinning, described previously as the first macroscopical sign of histolysis, is the result, no doubt, of the next histological stage to occur. In this stage the area of histolysis (*X*, fig. 3) has greatly increased, resulting in an actual decrease in thickness of the skin. This decrease is at the expense of the stratum spongiosum and stratum compactum; the subcutaneous connective tissue and epidermal layers being practically unaffected. Large numbers of lymphocytes have by now been attracted to the area and the dissociated elements of the histolyzed tissues are being rapidly removed, presumably by the lmphocytes. It is important to note at this point, also, that the lower portions of the stratum compactum undergo histolysis first and that there is a tendency for the upper portions to be pressed up against the epidermis, so that they assume the position formerly occupied by the stratum spongiosum.

This thinning process progresses rapidly (fig. 4), and in time only a narrow band of partly histolyzed stratum compactum is left beneath and close to the epidermis. The subcutaneous connective tissue and epithelial lining generally disintegrate at about this time or a little before, and shortly afterward the lower layers of the outer epidermis are affected. The final stage in the process is represented in figure 5 in which only a single layer of the outer epidermis (the cuticle) and a few strands of the stratum compactum remain. At this stage the skin is nearly transparent, viewed externally. Further histolysis results in a perforation, or the pressure of the fore limb may be sufficient to rupture the skin at this point and so initiate the perforation. In the absence of pressure supplied by the fore limb, further histolysis of the outer epidermis so weakens this layer that the tension of the surrounding normal integument is sufficient to cause a break in the layer and so form a perforation.

3. *Enlargement of the perforation*

The initial perforation rapidly enlarges as histolysis progresses in the surrounding skin. The epidermis at this time soon bends downward and covers the ragged edges of the perforation. Within one or two days after the appearance of the perforation, it has united with the epidermis of the fore limb at a point where the latter has previously made connections with the skin of the operculum (fig. 6). At this time the entire central portion of the 'shelf' is rapidly undergoing histolysis and consists of a heterogeneous mass of dissociated dermal elements heavily invaded by lymphocytes. It is interesting to note here that the epidermis surrounding this histolyzing mass is apparently very little, if at all, affected.

The enlargement of the perforation, therefore, is at the expense of this perforation edge or 'shelf.' Sections made through more advanced perforations present an appearance represented in figures 7 and 8. In the former the 'shelf' has undergone considerable atrophy, while in the latter it is absent entirely, although the fusion point with the fore-limb skin is clearly marked at *E*. A still later stage (fig. 9) shows a decided lessening of the 'notched' appearance at the fusion point. It is this slight irregularity in the epidermis, however, which is responsible, macroscopically, for the outline of the former perforation which persists for some time after the metamorphosis of the tadpole.

4. Union of the various layers of the opercular and fore-limb integuments

During the atrophy of the perforation edge the various dermal layers of the fore-limb and body integuments are not continuous, but seem to be broken up into their component elements at the point of fusion. This area is always heavily crowded with lymphocytes.

One of the most constant features is the presence of connective-tissue-forming elements (*C*, fig. 6) which occur in the subcutaneous connective tissue just below this area. Their function seems to lie in connecting the stratum-compactum layers of the fused integuments at this point. In figure 8 they can be seen definitely bridging the gap, which results finally (fig. 9) in the complete continuity of this layer. The stratum-spongiosum layer of the fore-limb skin is generally lacking at first, but appears at this time, or a little before, and is formed between the compactum layer and the epidermis which have up to this time closely approximated each other.

Soon after the establishment of a continuity in the stratum-compactum layer, the stratum spongiosum, so formed, unites with that of the opercular skin. This results in the complete continuity of all layers of the body and fore-limb integuments. A considerable number of lymphocytes remain for some time afterward, but eventually disappear.

SPECIFICITY OF THE INTEGUMENT

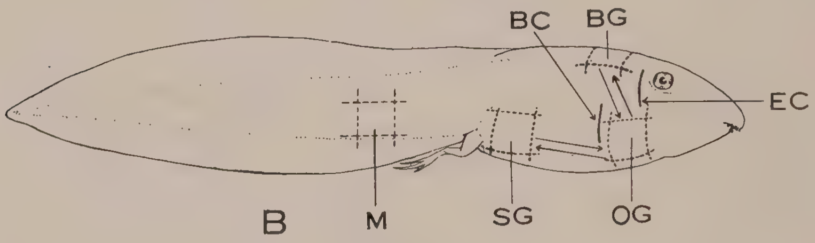
1. Purpose of the skin transplantations

The fact that histolysis normally occurs in a certain definite area of opercular integument would lead one to suspect a local specificity of that integument toward the histolytic agent or agents. This view is further strengthened by the 'outlining' of skin destined to undergo histolysis, which occasionally occurs. In order to determine whether or not this view is correct, it was decided to transplant skin autoplastically from regions of the body which normally do not undergo histolysis during metamorphosis to the perforation region. The matter of the possibility of local factors in the opercular region operating to destroy the skin was also tested by transplantation of opercular skin, which normally histolyzes, to other regions of the body.

2. Technique of skin transplantation

The technique employed in making skin transplantations was as follows: The anaesthetized animal was placed upon a damp cloth and a square or other rectangular incision made around the area of the skin to be grafted. The skin could then be stripped off with a forceps from the underlying tissues.

The grafts were taken from three regions, viz.: 1) from the right side in the region of the pectoral girdle where the fore leg appears during metamorphosis; 2) from the right side just anterior to the hind leg, and, 3) from the back just posterior to the eyes (text figure B, *OG, BG, SG.*). In making the transplantation, a rectangle of integument was removed from the region where the graft was to be placed and the latter fitted to this area, under a binocular microscope. It was found advisable to carefully flatten out the edges of the graft by running the point of a small spearhead scalpel around and under the peripheral edges of the transplant. This precaution prevented the edges of the graft from curling up underneath, and so facilitated the healing process.



Text figure B

3. *The healing process*

The transplanted graft of integument rarely covered the entire wound area that had been exposed by the removal of the transplant with which it was interchanged. This was chiefly the result of two factors, namely, the immediate post-operative tendency of the wound area to expand, the result of tension exerted by the surrounding integument, and, secondly, the tendency of the graft to contract when severed from its connections. The interchanged grafts being of the same size, it is clear that under these conditions there would exist a well-defined wound area separating the graft from the surrounding integument. Furthermore, it was essential for the experiment in hand that this wound area should heal over completely before the animal underwent metamorphosis.

The factors involved in the healing of such a wound have been discussed by a number of investigators, and will not be gone into here. In this connection the reader is referred to the works of Peters('85), Barfurth ('91), Loeb ('98, '02), Harrison ('10, '14), Holmes ('13, '14), Oppel ('13), et al. The time required by an autotransplant in uniting with the host skin[2] varied between wide limits. In some cases where the transplant filled the entire wound area, only four or five hours were required for the epidermis to become continuous. Usually, however, as previously explained, a well-defined wound area separated the transplant from the host skin, the covering of which required a period varying from twelve to forty-eight hours, depending on the width of the wound area. The epidermis which first migrates over this area is derived from cells moving centrifugally from the transplant edges and from similar cells moving centripetally from the edges of the host skin. The cells of the latter move with greater rapidity than those of the former, resulting in a fusion line located nearer the transplant than the host skin. This fusion line becomes sharply outlined in two or three days and, according to Cole ('22), is the result of an hypertrophy of epidermal cells. Just how long a period is necessary for the dermis of the two integuments to establish connections was not determined. That it requires less than three weeks was quite evident, since all grafts were sectioned by the end of this period, at which time no histological demarcation between transplant and host skin could be detected.

4. *Macroscopic changes in appearance of grafts*

The pigmentary changes which occurred during and after the healing process vary in respect to the type of graft concerned. Generally, the entire graft assumes a decidedly pink coloration within one or two days after transplantation. This is probably due to a congestion of the capillaries. This condition is purely transitory, however, and disappears in a day or two.

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[2] 'Host skin' refers to the normal undisturbed integument of an animal surrounding a skin-graft transplant or to any integument of an animal which has not been transplanted to another region of the body, but has been simply laid open for the reception of a graft, such as a limb-bud or gill transplant.

Back grafts, when transplanted to the opercular region, retain their original deep black pigmentation, and in many cases seem to attain a deeper shade than the normal integument of the back. Conversely, opercular grafts transplanted to the back had a tendency to assume a lighter shade. Both of these phenomena were particularly evident in R. catesbeiana grafts, and to a lesser extent in those of R. clamitans. Such observations were not illusory in nature, since adequate methods were employed when comparing the graft with integument from the area from which it was previously removed. This was accomplished by placing a piece of cardboard, containing a hole for observational purposes, over the graft so that the surrounding host skin, which might introduce illusory shading of the graft, was blocked off when making the comparison. Eventually, however, both types of grafts tended to assume the degree of pigmentation peculiar to the host skin, although exact correspondence of shading was never attained.

Two species were used in the skin-graft experiments. R. catesbeiana tadpoles were found to be most suitable in regard to the facility with which grafts could be removed and transplanted. Owing to the extreme difficulty in initiating metamorphosis artificially, however, work on this form was abandoned after more than a hundred operated animals failed to metamorphose completely. When about half metamorphosed and just before histolysis of the opercular skin occurred, an edematous condition invariably supervened, followed shortly by death or at best by a cessation of further development. Jordan and Speidel ('23) have given this condition the name of 'metamorphic stasis' and believe it to be the result of an inadequate development of certain blood-forming organs, notably the spleen, with the consequent deficiency of blood cells so necessary during metamorphosis.

More favorable results were obtained, however, with R. clamitans tadpoles. Two types of autoplastic transplantations were made, i.e., 1) opercular and back grafts interchanged, and, 2) opercular and side grafts interchanged. In addition, grafted control animals were employed for each type of operation. The control operation consisted in the removal and replacement of skin grafts to the same location in their original orientation. Controls of this type were employed in order to determine whether or not the effects of the operation (i.e., trauma or injury to the transplant) had any bearing on the behavior of the latter during metamorphosis.

The mortality rate encountered in series SC amounted to 56 per cent, twenty-eight out of fifty grafted animals dying at various stages of metamorphosis prior to the limb-perforation stage. Of the twenty-two successful cases, thirteen side-skin grafts exhibited 'thinning' and, with the exception of one case, formed perforations, while nine back-skin grafts exhibiting 'thinning' resulted in the formation of six perforations.

The four grafted control animals that survived metamorphosis exhibited thinning of the opercular graft somewhat earlier than was generally the case with the experimental animals. It was also quite evident that 'back' grafts of the latter exhibited thinning a day or two later than 'side' grafts. In both cases, however, the extent and location of the perforations were quite uniform and resulted in the destruction of that portion of the graft included in the histolytic area[3] of the normal animal. Figure 15 represents a typical case of a perforation involving about 50 per cent of a side graft. The history of this case (*SC 2*) is given in an abbreviated form as follows:

April 23, 1924. Autoplastic side graft placed in perforation region on right side. Normal integument from this region grafted to wound area exposed by removal of side graft.

April 23–30. Fed Spirogyra.

April 24. Both grafts light pink in color.

April 30. Both grafts normal in pigmentation and coloration.

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[3] 'Histolytic area,' the area of opercular integument which usually presents macroscopic signs of histolysis during the formation of the fore-leg opercular perforations. This area may or may not be sharply marked off visibly from the surrounding opercular integument.

April 30. Grafts fully healed. Animal placed in di-iodotyrosine solution for three days.

May 8. Tadpole rapidly metamorphosing.

May 8. Slight thinning on spiracle side. Slight thinning on right side involving anterior-ventral third of graft and skin below.

May 9. Small perforation at anterior-ventral edge of graft. Elbow of fore limb protruding.

May 10. Right fore limb released through perforation.

May 11. Perforation rapidly enlarging, involving thin areas of graft and host skin.

May 12. Elbow of left fore limb protruding through small perforation. Perforation on right side included 50 per cent of graft. Tadpole half metamorphosed (tail partially resorbed, hind legs 22 mm. long, cranium frog-like). Animal killed and both grafts with surrounding host skin preserved for sectioning.

Several back and side grafts were sectioned following formation of the perforation. Since the perforations were generally bounded posteriorly by graft tissue and anteriorly by host skin, it was possible to compare the histological picture presented by each from the same section of tissue. The portion of graft bounding the perforation showed clearly that typical histolysis had occurred which was identical in every essential to the histolysis of the host skin. Back grafts, in general, exhibited a much narrower zone of histolysis than side grafts (fig. 10). The presence, however, of disintegrating dermal layers heavily invaded by lymphocytes proved the difference to be purely a quantitative one and in no way dissimilar from the histolysis found in a normal perforation.

Two interesting cases were found in connection with this series. The grafts that had been transplanted to the histolytic areas of SC 10 and SC 40 were both lost before healing had set in to any extent. The surrounding host skin, however, succeeded in both cases in completely covering the large wound areas so exposed. Upon metamorphosis, perforations were formed in this newly formed integument which upon sectioning[4] proved to be the result of histolysis. Since the opercular integument previously removed included all of the skin which normally histolyzes, the result was the same as if integument adjacent to the histolytic area had been transplanted to that region. These two cases constitute, therefore, a third type of transplant and confirm the results obtained with the grafted animals.

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[4] 'Sectioning a perforation,' this is perhaps an ambiguous way of describing the sectioning of integument surrounding a perforation. Since it facilitates the localization of the particular area sectioned, however, its usage will be continued throughout this paper.

The only changes observed in opercular grafts transplanted to the back and side were those of pigmentation. Six of the twenty-two opercular grafts, so transplanted, were sectioned. No signs of histolysis were found, all layers of the dermis being normal, though this same integument, if it had been allowed to remain in its former opercular location, would have undergone histolysis.

INFLUENCE OF THE FORE LIMB

A. Extirpation of the right fore limb

1. Purpose of the experiment. The experiments involving skin transplantations to the opercular region showed quite clearly that any integument placed in the histolytic area will undergo histolysis during the metamorphosis of the tadpole. This fact logically suggests that the causative histolytic agent or agents concerned must be located in the immediate neighborhood of the disintegrating skin. The fore limbs seem to fulfill every requirement as regards location, constancy of position, and time of development. Reference to text figure A and the detailed account given in the introduction to this paper will emphasize this latter point.

Obviously, extirpation of the fore limb at various stages of development would throw light on its relation to opercular histolysis during metamorphosis as has been studied by Braus ('06) on Bombinator larvae. Should histolysis fail to occur in the usual manner, in the absence of the fore limb, it would signify that the latter is directly concerned in the process. Three series of limb-extirpation experiments were made on as many species of tadpoles, viz., R. pipiens, R.

sylvatica, and R. clamitans, in order to determine whether the reaction here was similar to that found by Braus for Bombinator larvae in which small perforations were formed during metamorphosis in the absence of fore limbs. In all three series only the right fore limb was removed, the left fore limb being left undisturbed to serve as a control.

2. Technique of limb extirpation. The technique found most suitable and productive of the lowest mortality rate in the extirpation of the right fore limb was as follows: The anaesthetized tadpole was placed upon a damp cloth and a dorsoventral incision made through the skin with a pair of finely sharpened thumb-spring scissors. This incision was made, in general, about 2 to 4 mm. posterior to the eye and from 3 to 5 mm. in length (*EC*, text figure B). In making the extirpation, the base of the fore limb was first located under the dissecting microscope, using a strong direct light, after which it was pulled out toward the incision by means of a fine-pointed and slender forceps. The limb, together with a small portion of the shoulder-girdle from which it arises, was then excised by means of a fine-pointed pair of scissors. After excising the limb, the edges of the incision were pulled together and the animal placed on its side in shallow water to heal and recover from the anaesthetic.

3. Extirpation of fore limb of R. pipiens. The animals used in this series were fully grown R. pipiens tadpoles with body lengths ranging from 50 to 70 mm., and hind legs of 15 to 30 mm. With the exception of a few cases, it was found unnecessary to induce metamorphosis artificially. Most of the tadpoles collected were in early stages of metamorphosis, and within three weeks underwent a normal transformation.

The extirpated fore limbs varied from 2 to 8 mm. in length, the average being 5 mm. Such limbs were of too small a size to exert pressure and so cause bulging of the integument. Their size was sufficient, however, to make their location and extirpation a simple matter. Of the thirty-odd specimens operated upon, eighteen successfully passed through all stages of metamorphosis. The greatest mortality was experienced within two or three days following the operation, and was due, no doubt, to postoperative effects.

The eighteen cases completing metamorphosis exhibited a striking uniformity as regards the histolytic changes which occurred in the skin of the opercular region. In all cases a definite and pronounced thinning occurred, which resembled, in points of area covered and time of appearance, the events taking place on the left side where the limb was left undisturbed (fig. 22). In three animals the areas were definitely outlined before thinning took place. Although the thinning process presented no great differences as compared with the left side, the same cannot be said of the perforations formed. The sixteen perforations formed varied in size and time of appearance and were not very closely correlated in either of these respects with the perforations formed on the left side. In general, however, a small hole, 0.5 mm. in diameter (*P*, fig. 22), appeared at the posterior end of the thin area within two to four days after the left side had perforated and released its fore leg. In most cases this minute perforation enlarged anteriorly until it reached a diameter of 2 to 3 mm. Several attained considerable proportions, being 4 to 5 mm. in diameter (*P*, fig. 18). In these latter cases the highly vascular atrophying gills could be seen protruding through the perforations. Case EP 6 represents, as far as possible, the average history of series EP animals. Abbreviated notes from this case are given below:

July 25, 1924. Right fore limb extirpated. Left fore limb undisturbed.

July 29. Tadpole showing distinct signs of metamorphosis.

August 4. No macroscopic indications of thinning on either side as yet.

August 5. Pronounced thinning on left side.

August 6. Left fore limb released through perforation formed in histolytic area. Small thin area developed on right side.

August 7. Thin area of right side slightly enlarged.

August 8. Thin area of right side considerably larger.

August 9. Small 0.5-mm. perforation present at posterior end of histolytic area.

August 10. Perforation enlarged to 2 mm. in diameter. Animal died. Sitting posture, pigmentation, cranial development, etc., typically frog-like. Tail a 10-mm. stump.

Several of these perforations were sectioned and the histological picture compared with that of similar sections made through perforations of the left side. Comparison was also made with the histological appearance of normal right-side perforations occurring in the control animals whose fore limb had not been removed. It was evident that a normal process of histolysis had preceded the appearance of an actual perforation in all cases. This fact was in direct agreement with the macroscopic signs of histolysis (i.e., thinning and outlining) that had previously been observed. Figure 11 represents a section through the edge of one of the perforations formed in the absence of the right fore limb. It will be noted that the dermis and subcutaneous connective tissue present essentially the same type of disintegration and an invasion of lymphocytes was found as in the formation of a normal perforation.

In three cases (EP 10, EP 19, and EP 21) small perforations developed within nine or ten days on the right side and remained quite small for a day or so, after which they were healed over by the surrounding host skin. All three of these perforations occurred at a rather late stage of metamorphosis and at a time when perforations are normally obliterated. This fact may account, therefore, for their small size and short duration.

4. Extirpation of fore limb of R. sylvatica. Series ES animals consisted of fully grown R. sylvatica tadpoles ranging from 50 to 56 mm. in length, and with hind legs ranging from 10 to 18 mm. in length. Such specimens were on the verge of metamorphosis and so required no artificial stimulation to induce the process. R. sylvatica tadpoles are noted for their rapid metamorphosis and, in fact, no cases were found in which more than eight days were required for complete transformation. The average period required was about five days, although some specimens completely metamorphosed within four days.

The extirpated right fore limbs averaged 5 mm. in length and had not attained that degree of development which results in a bulging of the opercular integument. The extirpation of such limbs involved less difficulty than in the case of R. pipiens or R. clamitans, due to the absence of bleeding during the operation. The animals were so little affected that it was found unnecessary to employ an anaesthetic during the operation, although anaesthetization made possible the exposure of the extirpation incision to the air following the operation, and so facilitated the healing process.

Nine cases of complete extirpation survived the period of metamorphosis. All of these developed definite thinning of the right-side opercular integument, at approximately the same time thinning was evident on the left side. Two cases of outlining were found. The extent of the histolytic area appeared normal on the extirpated side and in no way different from the histolytic area of the left side. In all, seven perforations occurred. These were first visible as minute holes in the center of the histolytic areas, and in no case exceeded 2 mm. in diameter as enlargement took place (*P*, fig. 16). Perforations of the left side generally appeared about twenty-four hours before the initial appearance of those on the right side, and while still quite small made possible the releasing of the left fore limb. Considerable enlargement occurred after the fore limb was released, however, so that they generally equaled twice the diameter of the right-side perforations. The latter, after reaching a certain size, appeared to remain unchanged for a period of one or two days, after which processes of healing became evident. The following condensed notes from the case history of ES 8 will serve to exemplify the typical sequence of events in the metamorphosis of series ES animals:

June 16, 1924. Right fore limb extirpated. Limb length, 5 mm.
June 18. Fed Spirogyra from this date on.
June 19. Extirpation cut apparently fully healed.
June 20. Thin areas present on both sides.
June 21. Elbow of left fore limb protruding through small perforation on left side.

June 22. Small perforation present in thin area of right side. Left fore limb released through perforation on left side, which is twice as large as the right-side perforation.

June 23. No significant changes. Animal typically frog-like.

June 25. Animal same as on June 23rd. Died. Preserved for sectioning and histological study of perforation region.

The sections through the right-side perforations of series ES animals presented essentially the same histological picture as found in sections through R. pipiens perforations. It was quite apparent that the formation of the perforations was the result of an histolysis of the integument of that region, the histolysis having occurred in the complete absence of the fore leg.

5. *Extirpation of fore limb of R. clamitans.* The R. clamitans tadpoles used in this series were extremely large, second-year forms, with body lengths varying from 75 to 105 mm. (average length about 95 mm.). The average hind-leg length was about 30 mm. These specimens appeared to be in an early stage of metamorphosis, their future behavior proving this to be the case, since no artificial agents were required to accelerate their development. The fore limbs, at the time of operation, were quite well developed and measured from 7 to 10 mm. in length. The location of the limbs was easily determined, since at this stage a slight bulging of the opercular integument was evident, due to their presence. That histolysis had not begun in the opercular skin under pressure was determined by a histological examination of several specimens.

Eleven operated animals metamorphosed in periods varying from two to five weeks. The metamorphosis was very complete, however, the tympanic membrane stage[5] being reached in all cases. The left fore leg was released in all cases through perforations which formed within ten days following the extirpation of the right fore limb. Despite the complete metamorphosis, only five cases of thinning appeared on the right side, none of which developed into perforations. Two of these cases were associated with a distinct outlining of the histolytic area, one of which (EC 3) was sketched (*OA*, fig. 19). Sections through the thin areas so developed showed that histolytic processes were at work, but were developing at a very slow rate. Several animals were sectioned which exhibited no external signs of thinning. No histological signs of histolysis were detectable in such cases, the integument appearing normal in every respect.

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[5] 'Tympanic membrane stage,' the formation of the tympanic membrane constitutes one of the last external structures of the frog to be developed during the metamorphosis of tadpoles. This stage of metamorphosis is seldom reached in artificially metamorphosed animals.

B. Fore-limb transplantations

1. Purpose of the experiment. The fact that histolysis and perforations occur in the opercular integument in the absence of the fore limb does not rule out the possibility that the latter may be concerned in the process when present. In fact, there were strong indications that the rate of histolysis during metamorphosis was at least retarded in R. clamitans when the fore limbs were removed.

An experiment designed to answer this question consists in the transplantation of the fore limb under the normal integument of other regions of the body than the operculum. In order for the experiment to be significant, however, the transplanted fore limb must acquire an adequate blood supply in its new environment, and so establish itself that growth and development approximating that in its normal position will occur.

Since preliminary experiments had shown the inability of fore limbs to heal in readily when differentiated to any extent, it was finally found necessary to confine the transplantations to limb-bud stages. The most suitable material at the time consisted of R. palustris tadpoles. Their fore-limb buds could be easily transplanted, and although only homoplastic grafting was possible, a fairly high percentage of successful grafts resulted. Autoplastic grafting, although possessing distinct advantages for the experiment in hand, was not possible, due to the 100 per cent mortality encountered after the removal of the fore-limb buds.

2. *Technique of homoplastic limb-bud transplantations.* Homoplastic limb-bud transplantations involved the removal of the right fore-limb bud of one tadpole and its transplantation to the musculature of the back of another individual. For isolating the limb bud, a large incision was made in the pectoral region of the right side and a considerable portion of the shoulder-girdle, containing the limb bud, together with adhering muscle, was removed. This was placed in a watch crystal with a few drops of blood serum and fluid taken from the opercular cavity. The bud was then dissected free of all extraneous tissues and most of the shoulder-girdle, so as to leave only a small portion of the latter attached to the bud. The buds so extirpated, ranging in size from 0.3 to 0.7 mm. in length, were still in an undifferentiated condition.

Two adjacent sides of a 6-mm. square of skin were next cut just posterior to the eye on the back of a second tadpole (*C*, fig. 21). The incisions so made allowed half of this square of skin to be laid back over the other half. A small slanting incision was then made in the underlying musculature just to one side of the middorsal line and at a point close to where the skin was folded back on itself. Into this incision the limb bud was inserted, shoulder-girdle end first, until only about half of the bud protruded above the level of the muscle. The flap of skin was then laid back, the edges smoothed out flat, and the animal placed in shallow water to heal and recover from the anaesthetic.

3. *Homoplastic limb-bud transplantation in R. palustris.* Series LT host animals consisted of R. palustris tadpoles ranging from 30 to 45 mm. in length, and with hind leg lengths of 2 to 10 mm. It was quite evident that growth was proceeding very slowly in these specimens, since little development took place in stock animals maintained under laboratory conditions for a month or more. Consequently, in all operated animals it was necessary for the purpose of the experiment to induce metamorphosis artificially. Solutions of di-iodo-

tyrosin were found to accelerate the metamorphosis of this species in a satisfactory manner. The time required for complete metamorphosis to occur varied, however, within wide limits. The average period required was ten days after immersion in the di-iodotyrosin solution. On the other hand, a period as long as eighteen days was required by some larvae to reach the stage of metamorphosis desired. This stage may be described here as that at which the fore limbs are normally released through the opercular perforations. This stage was successfully passed by the large majority of operated animals, the greatest mortality occurring one to two weeks after the stage was reached. The late stage at which death occurred, therefore, was very favorable for the experiment in hand.

The homoplastic limb buds were secured from less developed specimens as compared with the animals to which the transplants were grafted. The limb buds of this particular stock appeared to differentiate shortly after a length of 1 mm. was reached. Consequently, all buds used for transplantation purposes were generally less than 1 mm. in length, the average length being 0.5 mm. Tadpoles possessing fore-limb buds of such a size rarely exceeded 35 mm. in length, the hind legs being generally present as buds 1 mm. long. As previously described, the host animals, to which the fore-limb buds were grafted, were considerably more developed, the hind limbs always possessing some degree of differentiation.

Eighty-four homoplastic transplantations were made. Of these, seventy-five animals were successfully metamorphosed to the stage where the fore legs of the host animal were released through the normal perforations forming in the operculum. The percentage of successful grafts was quite high for homoplastic transplants, being 33 per cent. This percentage was based on twenty-five cases in which the limb bud had undergone growth and differentiation. Since they constitute the only cases of series LT which were of value in answering the questions for which the experiment was designed, the remaining fifty cases will not be discussed further.

During the early stages of differentiation, the growth of the transplants was not detectable externally, since no pigmentary changes or bulging of the integument occurred. As development progressed, however, the rapidly growing fore limbs exerted pressure against the integument of the back so that a slight bulging of the latter was in time distinguishable. The amount of bulging varied considerably in different cases and in a rough way indicated the relative size to which the fore limb had developed beneath the integument.

The area of integument under pressure generally assumed a lighter coloration, which was easily distinguished from the deep black pigmentation of the surrounding skin. This change could not be compared, however, to the macroscopic appearance of skin undergoing histolysis which has previously been spoken of as appearing 'thin.' The latter condition of the integument is always the result of a definite decrease in thickness of the skin, the result of histolysis. In a few cases of very strong bulging, however, a slight decrease in thickness was noticeable, due, no doubt, to the tension of the skin. In such cases the lightness of coloration may have been somewhat accentuated as a result.

Although the integument of the back was under considerable pressure in the majority of cases for periods ranging from five to twelve days, only three cases of perforation appeared. All three of these were preceded by very strong bulging of the integument and appeared to originate at a point where the elbow of the transplant was exerting considerable pressure. In fact, the latter was always found protruding through the perforation as soon as it was formed. Two of these cases were preserved for sectioning as soon as the perforations occurred, while the third was allowed to develop. Within a day after the initial appearance of this perforation, the fore limb was released (figs. 21 and 21 A). The releasing of the latter resulted in the disappearance of all signs of bulging, the surrounding integument resuming its original normal condition in this respect. Although the specimen lived for several days following the release of the transplanted limb, no increase occurred as regards the size of the perforation, the edges of the latter appearing to adhere closely to the integument of the fore limb. The specimen was finally preserved for histological study.

The three perforations, so formed, were sectioned and examined for histological signs of histolysis. It was evident at once that the perforations had not been preceded by an histolytic process. There were no signs of disintegrating dermal elements present, although a small number of lymphocytes seemed to have been attracted to the area. The presence of lymphocytes was to be expected, since they are usually present in tissue under unusual pressure or irritation. Figure 12 represents a section made through one of the perforations preserved as soon as it had appeared. It will be noticed that the various layers of the integument are continuous to the very edge of the perforation, where they show unmistakable signs of having been ruptured. Several other cases were sectioned which, although no perforations had formed, exhibited even more pronounced bulging of the integument than was true of the perforation cases. In none of these sectioned specimens could any marked histological changes be observed which might be interpreted as constituting even a very early stage of histolysis.

C. Glass-bead implantations

1. Purpose of the experiment. The fact that three perforations were formed by the developing fore-limb-bud transplants, which histologically presented no evidence of having been preceded by an histolytic process, suggests that pressure alone may have been responsible for their occurrence. To test this we might make use of any solid object, so placed in the organism as to exert considerable pressure against the integument. Furthermore, the use of a non-living object would rule out all possibility of any agent other than pressure being in operation. Consequently, glass beads were selected for the purpose. Cole ('22) successfully used this method in determining the reaction of artificial eyes of glass and celloidin on tail and back skin grafts placed over them. Absorption of tail skin grafts was found to occur to such an extent that the artificial eyes were liberated. The histological process of the absorption of the grafts was not described, however.

2. Technique of glass-bead implantations. The tadpoles used in the bead-implantation series were first anaesthetized in the usual manner by immersion in a solution of chloretone. They were then placed on their side on a damp cloth and a 5- to 6-mm. vertical incision made through the integument of the right side of the pectoral region. This cut was in general somewhat posterior to the incision made for limb extirpation (*BC,* text figure B), but differed in that only the integument was affected, care being taken not to injure the underlying periotoneum. Next, a band of skin was carefully loosened from the periotoneum as far back as the hind limb of that side. This was accomplished by inserting a small, flat, spearhead scalpel under the integument from the incision previously made and carefully cutting the fine connections which bind the integument to the underlying periotoneum. Extreme care was necessary in order that tearing of the peritoneum, which is comparatively thin and delicate along the sides, did not occur.

The glass beads employed were not spherical in shape, but slightly flattened on two sides. The implantation was done by slipping the bead under the skin through the incision previously made in the pectoral region. It was then an easy matter to push the bead back to its desired location just anterior to the hind limb. The edges of the cut were then drawn together and the animal allowed to recover from the effects of the anaesthetic.

3. Bead implantation in R. clamitans and R. pipiens. Two species of tadpoles were used in the bead-implantation experiments, namely, R. clamitans and R. pipiens. The former species consisted of individuals ranging from 50 to 90 mm. in length. The smaller members were quite undeveloped, as evidenced by the small size of the hind limb, while the larger specimens were considerably more advanced and were evidently entering upon the early stages of metamorphosis. The R. pipiens tadpoles used were more uniform in size and stage of development. They varied between 55 and 75 mm. in length and possessed, in practically all cases, well-developed hind limbs varying between 16 and 32 mm. in length. R. pipiens tadpoles of such dimensions are quite definitely recognized as being in an early stage of metamorphosis and required no artificial stimulus, therefore, for the completion of that process.

The mortality rate was comparatively low, not exceeding 15 per cent in either the R. clamitans or R. pipiens series. This was no doubt due to the simple type of operation performed, and also to the fact that the animals were not subjected to artificial methods for inducing precocious metamorphosis. A considerable number of operated animals failed to be of value, however, in that the bead transplant often shifted its position, assuming a midventral location in some cases, while in others the migration proceeded anteriorly. In the latter case the beads frequently escaped through the partially healed incision. All cases in which the bead tended to change its position were discarded.

Five cases of bead implantation in R. clamitans were obtained in which the bead remained in place for at least ten days. Two of these developed perforations. Of these animals, one (fig. 20) was quite fully metamorphosed, both fore legs having been released fully a week prior to the appearance of the bead perforation, which was first evident on the twenty-fifth day following transplantation. The other (fig. 17) had undergone only slight transformation, as evidenced by the lack of fore-limb development and the presence of the typical tadpole tail. The perforation in the latter case appeared on the twelfth day following implantation of the bead. The remaining three individuals failed to develop perforations, although the integument was bulged out strongly for over three weeks in each case.

The R. pipiens series reacted more favorably than the R. clamitans series. Fifteen successful cases of bead implantation were obtained which, with one exception, developed perforations. The time of appearance of the perforation varied within wide limits, four appearing within one day, four within two days, one within three days, two within five days, and three within seven days following the operation. The time of appearance of the perforation was in no way correlated with the metamorphic stage of the animal. Figure 17 represents animal BT 11 which developed a perforation while still in an early stage of metamorphosis.

Several specimens were sectioned just prior to the stage when the bead perforation was due to appear, in order to determine the histological cause of the translucency and grayish coloration of the integument under pressure. Such sections showed that the only change in the integument was a decrease in thickness. This was not due to an histolytic process, however, since all of the cellular layers were intact, but to a stretching of the integument as a whole. Several perforations in various stages of enlargement were also sectioned and examined histologically. The integument bounding the edges of the perforations showed clearly that no histolytic processes had been concerned in their formation. The various layers of the integument were in all cases continuous to the edges of the perforation, where signs of rupturing were plainly evident. This was especially true of sections made through perforations which had just formed. In more advanced perforations, where enlargement had ceased, the edges tended to round off. Histologically, this was seen to be due to the tendency of the epidermis to cover the ruptured edges of the various layers by a downward growth (fig. 13). In such cases, large numbers of blood cells, chiefly lymphocytes, were often present just below the epidermis. This fact, however, could not be construed as lending evidence to the possible presence of an histolytic process, since the blood cells were always confined to a rather limited area and were never found to any extent invading the dermal layers. Evidently, they had been attracted to the point of injury and were concerned chiefly with processes of repair. Had histolysis been in operation, they would certainly have functioned as phagocytes and invaded the disintegrating layers. This fact has previously been emphasized in describing the histological events concerned in the formation of a normal opercular perforation.

HISTOLYTIC FUNCTION OF ATROPHYING GILLS

1. Purpose of the experiment

The experiments involving fore-limb transplantation and bead implantation have shown that the fore limb is incapable of initiating histolytic processes in the integument. These results are in close harmony with the results of limb extirpation, which show quite clearly that normal histolysis of the opercular integument may take place in the absence of the fore leg to as full a degree as when the latter is present. Finally, it has been shown by skin transplantations that the opercular integument of the histolytic area is not peculiarly specific in its ability to undergo histolysis, but is subject to that process solely on account of its location.

The various types of experiment described so far, while serving to answer a number of questions as regards the function of the fore limb during the formation of the opercular perforation, have signally failed to demonstrate the agent or agents responsible for inducing histolysis of the opercular integument during metamorphosis.

The clue to the agents later found to be directly responsible for histolysis was obtained from animal LT 40, a case of limb-bud transplantion. On the sixth day after operation in this animal, a small perforation was observed in the integument under which the transplant had been placed. Autopsy of the specimen later on showed that LT 40 was one of the fifty cases of the series in which the fore-limb bud failed to develop. Examination of the perforation under the binocular microscope showed the area to be filled with a white, fluffy mass, resembling gill tissue in all respects. Upon removing the integument, containing the perforation, a small mass of gill tissue was found lying on the musculature of the back. Fortunately, it had been transplanted accidentally when the limb-bud transplant had been made.

The occurrence of a definite perforation closely associated with gill tissue and in the absence of a fore limb immediately suggested the possible agents concerned, namely, the atrophying gills. The gills of anuran larvae undergo a striking and profound change during the metamorphosis of the latter. Although the writer has not made a histological study of such gills during this period of development, it is a well-known fact that they undergo a pronounced and rapid degeneration. They are characteristically of a deep reddish hue during metamorphosis, contrasting strongly with their normal pink or dark gray color, and in many cases may be seen protruding through the anterior portions of well-developed opercular perforations along with the fore legs. In the adult frog the gill remains have received more consideration. Here they occur as small, capsulated, gland-like structures known as the ventral branchial bodies or the so-called 'Kiemenreste.' Their function is as yet quite obscure, although there is a certain amount of evidence at hand to indicate that in their final state they are lymphoidal in nature.

Without going into a detailed discussion, it may be said that the atrophying gills of metamorphosing tadpoles presented a possible histolytic agent which possessed all of the qualifications which have previously been set forth as essential. Consequently, experiments were designed to find out whether they were, in reality, the active histolytic agents operating in the formation of opercular perforations.

2. Technique of gill-transplantation experiments

The technique involved in the transplantation of gill tissue was similar in many respects to that followed in the limb-bud series. In general, an entire gill or the distal halves of two gills were removed from one animal and transplanted, homoplastically, to the back of another. The gills so transplanted were in some cases normal gills, while in others they were degenerating, being taken from tadpoles undergoing or near the close of metamorphosis. In some instances the gill tissue was inserted into the back muscle of the host animal, while in others it was simply placed on top of the muscle.

In two series a square of integument was first removed from the back of the host animal, the gill tissue then transplanted, and the latter finally covered by a graft of opercular integument obtained from another tadpole. A third series consisted in the transplantation of gill tissue under the normal integument of the back, no skin grafting being necessary. The technique here was identical to that followed in the limb-bud series. An effort was always made to orient the gill transplant so that little or no bulging of the integument would result on account of the transplant.

3. *Homoplastic gill and skin transplantation in R. palustris*

Series GST consisted of homoplastic gill tissue and opercular skin grafts transplanted to the backs of host animals. The gill transplants were obtained from tadpoles exhibiting no visible signs of metamorphosis and in which the gills appeared to be normal in every respect. The opercular skin grafts were always taken from the area known to undergo histolysis during metamorphosis. Generally, both transplants were obtained from the right side, but in all cases, however, from the same animal. In grafting the gill tissue, the latter was, in the large majority of cases, transplanted into the musculature of the back so that only a small portion was in contact with the skin graft above. This was desirable in order that the pressure exerted against the latter by the gill transplant be reduced to a minimum.

In all, forty-eight operations were made. Of these, thirty-five cases succeeded in attaining a stage of metamorphosis adequate for the experiment in hand. The outstanding feature was the high percentage of perforations that were formed. In twenty-eight cases out of a possible thirty-five, perforations of various sizes developed in the skin grafts. The perforations always occurred directly over the transplanted gill tissue and in many cases rapidly enlarged to include fully fifty per cent of the skin graft. The time required by the perforation to develop varied between three and seven days. The large majority, however, appeared on the fourth, fifth, and sixth days following the operation.

The perforations were in all cases preceded by a definite process of thinning corresponding closely to the macroscopic thinning process previously described in connection with the formation of a normal opercular perforation. It should be mentioned here, also, that of the seven cases in which perforations failed to appear, four definitely presented thinning of the skin grafts. Perforations would undoubtedly have occurred in these cases had the animals remained alive for a day or two longer. The three cases in which no macroscopic thinning was visible were autopsied and the gill transplants found to have been inserted quite deep in the muscle, so that contact with the skin grafts had not been possible. Whether the absence of contact accounted for the failure of these grafts to undergo thinning was not determined.

Generally, a very slight bulge was evident in the skin graft, due to the presence of the gill transplant. The thinning was first noticeable in the integument over this bulge, and resulted in a loss of coloration, the area assuming a grayish, opalescent shade, one or two days before the perforation made its appearance. The appearance of the latter was not sudden, as in the case of the limb-bud-transplant perforations, but very gradual. An extremely minute hole, visible only under the dissecting microscope, first appeared. Its edges were regular in outline and presented no signs of having been ruptured by pressure. This minute hole steadily enlarged until it became visible to the naked eye. Although most of the perforations arose in this manner in the center or near the center of the skin grafts, their enlargement, in many cases, extended so as to involve fully one-half of the graft. The degree of enlargement was no doubt partially due to the amount of gill tissue present. The latter was always plainly visible, filling part or all of the perforation.

Several of the skin grafts were sectioned just before perforations were due to appear and examined histologically for signs of histolysis. It was at once apparent that an histolytic process, identical to that present in the normal opercular perforation was in operation. Histologically, the macroscopic appearance of thinning and opalescence was plainly due to a marked decrease in the thickness of the graft, the result of histolysis of the integument. Five perforations were also sectioned, which presented the same evidence of the activity of an histolytic process having preceded their appearance.

A series of sixteen control animals were metamorphosed along with the experimental animals of series GST. These controls involved the same operative technique employed in the case of the experimental animals, with the exception that gill transplantations were not made. Opercular skin grafts were transplanted, homoplastically, to the backs of tadpoles and the animals metamorphosed, in order to determine whether or not such grafts underwent disintegration in any way during the process. Ten controls were successfully metamorphosed. In all cases no macroscopic changes were observed in the skin grafts, while a histological examination of five of these later showed that the integument was normal in all respects and presented no changes which might be interpreted as constituting even the earliest stages of an histolytic process.

4. *Homoplastic gill transplantation in R. palustris*

R. palustris tadpoles from the same stock and of approximately the same size as those used in series GST were employed in series GT. The operation differed from the foregoing in that opercular skin grafts were not transplanted to the backs of the host animals. Gills showing no external signs of atrophy were simply transplanted homoplastically under the normal integument of the back. The latter was cut and turned back for the reception of the gill transplant in the same manner as in the limb-bud transplantations. With the exception of three cases, the gill transplant was always partially inserted in a small incision made in the musculature of the back.

Ten cases of successful metamorphosis were obtained in series GT. Of these, nine developed perforations of varying sizes which, however, were somewhat smaller on the average than those of series GST. To these nine cases must also be added the limb-bud transplant case LT 40, where a piece of gill tissue was accidentally transplanted and which represented the same type of operation, thereby bringing the total number of perforation cases to ten.

Thinning of the integument and the resulting opalescence, so typical in series GST prior to the appearance of the perforations, was much less pronounced in series GT, although generally detectable. This was due, no doubt, to the deep black pigmentation of normal integument of the back as compared with the lighter pigmentation of the opercular skin grafts. The perforations appeared, however, on the average, in about the same period of time following the transplantation of the gill tissue. This was to be expected, since opercular and back integument of R. palustris tadpoles are of approximately the same thickness and differ only as regards their pigmentation.

Several perforations were sectioned, including that of LT 40, and examined microscopically. As in the preceding series, their formation was plainly the result of an histolytic process. The one case in which a perforation failed to develop was also sectioned and histolysis found to be well under way. Had the animal survived another day, a perforation would surely have formed.

5. *Homoplastic gill and skin transplantation in R. pipiens*

The host animals in this series consisted of R. pipiens tadpoles with body lengths of 50 to 65 mm. and with hind-leg lengths ranging from 8 to 25 mm. Most of these were on the verge of metamorphosis, although retaining many typical tadpole characteristics. Although metamorphosis would have occurred normally in such specimens, all operated animals were placed in a di-iodotyrosin solution to accelerate the process. It might be well to mention here that the strength of the di-iodotyrosin solution was never such as to effect the general health of the animals or interfere with or influence in any deleterious manner the fate of the gill and skin grafts present.

The nature of the operation employed in this series was similar in most respects to that followed for the R. palustris GST series. In two respects, however, the operation differed markedly. The gill transplants were obtained not from normal gills as was hitherto the case, but from gills which had undergone considerable atrophy. In fact, all gill tissue transplanted in this series was obtained from tadpoles which had practically completed a normal process of metamorphosis and which were typically frog-like in appearance. The normal opercular perforations had in most cases practically disappeared, although in a few a small portion remained anterior to the protruding fore limb. Through this the atrophying gills were generally visible. The gills in such specimens were evidently nearing the close of the atrophying process. The gill transplant was always laid on the back muscles of the host animal and never inserted into them. An opercular skin graft was then placed over the gill transplant and fitted to the wound area. Since it was obviously impossible to secure the skin grafts from the histolytic area of the donor contributing the gill transplant, it was necessary to employ a third specimen for this purpose. The donors of the skin grafts were tadpoles in approximately the same stage of development as were the host animals, so that no changes had as yet occurred in the integument of the histolytic area.

Twelve animals were operated upon, eleven surviving a sufficient length of time to be of use. Eight cases of perforations developed, while the remaining three, although presenting marked macroscopical signs of histolysis, failed to perforate. The perforations developed with remarkable rapidity in this series, the average period required for their appearance being three and one-half days following the transplantation of the gill tissue.

The macroscopic changes preceding the appearance of the perforations were quite characteristic in this series and differed somewhat from those described in R. palustris. The differences were evidently due to a specific difference and to the fact that the atrophying gills were in a pronounced state of disintegration. In general, the skin grafts assumed a light reddish tinge within a day following their transplantation, as previously described in connection with the skin transplantation experiments. This always disappeared, however, within a day, after which the graft appeared quite normal. At least twenty-four hours before the perforation appeared, however, the portion of the graft lying directly over the gill transplant mass invariably assumed a light reddish tinge, and in some cases this later gave way to an opalescent shade.

The perforation always developed as a small hole located at some point on the slight bulge caused by the gill transplant. Its enlargement was both rapid and characteristic. The integument immediately surrounding the perforation was generally whitish or opalescent in color and consisted evidently of a narrow band of much histolyzed skin. Immediately surrounding this band was a second narrow zone characteristically deep red in color. The remaining portions of the skin graft presented varying degrees of coloration from a light pink shade to the normal color of the opercular integument. The initial perforation, so formed, enlarged in most cases so that fully 50 per cent of the graft became involved within a period of twenty-four hours. Figures 24 and 24A represent the typical appearance of a twenty-hour perforation. The continued growth of the perforation in several cases resulted in the histolysis of fully two-thirds of the skin graft, in which case the perforation reached and included the boundaries of two sides of the graft.

Several perforations were sectioned and examined histologically. Again it was at once evident that a typical process of histolysis had preceded their formation, similar in all essential respects to the condition found in the sections made through the perforations of the R. palustris series. The histolysis here, however, had evidently progressed at a much more pronounced rate, since many of the dermal layers presented a state of disintegration closely akin to liquefaction. This was particularly true of the stratum-compactum layer (fig. 14) in which the individual fibrils appeared to have coalesced more or less into solid masses, which were found distributed throughout the gill tissue which had been sectioned along with the histolyzing integument. The liquefaction of the stratum compactum was continued for quite a distance back from the edge of the perforation, as will readily be seen by consulting figure 14. A correspondingly large number of blood cells, chiefly lymphocytes, were also found distributed throughout the gill tissue and the disintegrating dermal layers of the skin graft.

DISCUSSION

The results of the skin-transplantation experiments (series SC) and of all other series involving the transplantation of integument, either autoplastically or homoplastically, have served to emphasize the ability of most integument to undergo histolysis when associated with the histolytic agents, the atrophying gills. There can be little doubt, therefore, that the integument of anuran larvae is not a self-differentiating tissue as regards the phenomenon of histolysis which occurs during metamorphosis. According to Braus ('06) and Schulze ('24), the opercular integument, which normally disintegrates and perforates during metamorphosis, should have responded in a like manner when transplanted to other regions of the body, if their supposition of a self-differentiating structure were correct. The failure of the opercular grafts to undergo these changes during metamorphosis is conclusive evidence, however, that such a self-differentiating property is not peculiar to the integument.

The question of local specificity of the skin has received considerable attention in the past, and the bulk of the evidence, it must be admitted, strongly supports the presence of such a specificity. Most of these experiments, however, have dealt with morphological characteristics of the integument rather than with physiological ones. Thus, the specificity of pigmentation has been shown by a number of workers to be quite localized. Winkler ('10), for example, transplanted the integument of Hyla and found that both belly and back skin retain their peculiar pigmentary characteristics. A little later, Weigl ('13) found that the markings and coloration of larval salamander integument are maintained when the latter is transplanted homoplastically. In the same year, Borst ('13) summarized all evidence up to that time relating to the specificity of protoplasm in general. The reader is referred to his paper for further details of investigation on this subject prior to 1913. More recently, Uhlenhuth ('17) has found that the yellow spotting of larval Amblystoma skin is maintained following homoplastic transplantation. He even finds that a particular type of yellow spotting will be preserved by a graft in the presence of a dissimilar type of yellow spotting peculiar to the host animal. Cole ('22) describes the preservation of pigment pattern and translucency of tail skin when the latter is transplanted to the back. The same was found to be true of back-skin grafts transplanted to the tail. He concludes, "there must be, therefore, in the integument of these regions, different substances which are constantly being produced by the cells and which determine the character of the new cells formed."

Although a local specificity may be present in the integument of the frog tadpole as regards its pigmentation and other more or less morphological characteristics, it does not follow that the same is true of certain of its physiological traits. In fact, we should rather expect to find that certain fundamental physiological reactions are characteristic of all integument irrespective of its location. This is shown by the present experiments in the common tendency of all integument to undergo a definite histolysis when associated with a certain causative mechanism. There seems to exist, however, a quantitative specificity of the integument which governs the rate at which the histolysis may progress. Thus it was found that while side skin, when transplanted to the opercular region, histolyzed in approximately the same time as normal opercular skin during metamorphosis, the same was not true of back skin. Such grafts invariably required a longer period of time to histolyze completely. This may have been due partially, however, to the relatively greater thickness of back integument as compared to either opercular or side integument.

An interesting situation exists in respect to the integument of the fore limb, which apparently is unaffected by the histolytic agents. It must be conceded, therefore, that the newly formed integument of the fore limbs is specific in this respect. It is in as close, if not closer, proximity to the same histolytic influences that initiate histolysis of the opercular skin, yet it remains unchanged. This peculiar situation might be likened to the tissues of the digestive system which, although secreting powerful proteolytic enzymes and the like, remain for the most part unaffected themselves, although constantly bathed in the latter. The explanation may also be found in the fact that the inner layers of the opercular integument are exposed to the histolytic agent, while only the outer layer or epidermis of the fore limb is thus exposed.

The fact that the left fore limb is released in the large majority of cases before the right fore limb might raise the question whether the histolysis of left opercular integument proceeds at a more rapid rate than is true of right-side opercular integument. Observations on over six hundred tadpoles undergoing both normal and artificially induced metamorphosis have shown that this is not the case, however.

The histolytic processes progress at the same rate on both sides and cannot, therefore, be concerned with the priority of left fore-limb eruptions. The probable explanation of this phenomenon would seem to lie in the asymmetrical condition of the operculum as regards the presence of the spiracle on the left side. The histolytic area is in close proximity to this natural perforation and hence the fore limb of that side is not required, in the large majority of cases, to erupt the weakened histolyzed integument, but may first appear through the spiracle. In any event, the mere presence of the spiracle in the histolytic area or close to it so weakens the integument that the fore leg is almost invariably released prior to the appearance of the fore leg of the opposite side. Speidel ('24) finds that if nearly full-grown tadpoles are immersed in a thyroid solution for two days, the original tendency of certain animals toward prior right fore-limb eruption is overcome. He furthermore finds that thymus treatment results in a somewhat greater than normal percentage of right fore-limb eruptions prior to the left. Swingle ('23) figures two hypophysectomized tadpoles metamorphosed with di-iodo-tyrosin, in which the right fore limb has appeared first. The phenomenon seems not uncommon in pituitariless larvae whose metamorphosis has been greatly accelerated by the administration of certain organic iodine compounds.

A consideration of the gills, as the active agents concerned in the histolysis of opercular integument during metamorphosis, presents several interesting features. In the first place, the results clearly show that the greater the degree of gill atrophy, the more potent are these structures in initiating integumentary histolysis. This was strikingly demonstrated by the transplantation of normal and atrophying gills beneath opercular skin grafts, the atrophying gills of metamorphosing larvae functioning with extreme rapidity as compared with the normal gill transplants. The normal gill transplants, however, undoubtedly underwent a certain amount of degeneration when transplanted, since the histolysis of the integument due to their presence cannot be interpreted in any other way. This will be seen to be true when we consider the inability of normal gills in the opercular cavity to induce histolysis of opercular integument.

The question of the nature and causation of autolysis has for a long time been the subject for numerous investigations and controversies. While it is not the purpose here to go into a detailed discussion of the problems presented by this phenomenon, it seems advisable to point out a few of the more fundamental points that have been established or are in controversy up to date, in order that the possible causation of opercular histolysis as described in the present paper may be more adequately interpreted.

A generalized definition of autolysis might be given as ''a degenerative tissue process involving the hydrolysis and liquefaction of the tissue proteins.'' Autolysis in its varied forms is quite universally distributed throughout the animal kingdom. While this fact has been well known by even the earliest investigators, the exact causative factors involved have been subject to widely different opinions. Two entirely diverse theories immediately arose which have both been strongly supported from time to time. Metchnikoff ('83) introduced one of these in his well-known phagocytosis theory. According to Metchnikoff, the leucocytes, and particularly the polymorphonuclear leucocytes, are the causative agents of tissue autolysis. This veiw has since been strongly supported by Noetzel ('95), Anglas ('04), Guieweysse ('05), and many others, the chief contention being that the leucocytes are able by their presence to initiate autolytic processes, presumably through the secretion of a chemical substance. It has also been thought that the presence of the leucocytes so irritates the tissue cells that the latter secrete an autolytic enzyme. The opposing theory which has gained considerable impetus during more recent years in connection with biochemical studies of autolyzing tissues, both 'in vivo' and 'in vitro,' claims that such is not the case. According to this latter theory, autolysis is the result of a chemical liquefaction of the tissue proteins, which have been catalyzed by enzymes present or forming in the cells of the tissue. The occurrence of leucocytes is interpreted as a purely phagocytic phenomenon and therefore of secondary importance.

The evidence advanced in support of Metchnikoff's theory is largely histological in nature and is based chiefly on the fact that large numbers of leucocytes are always found in the area of tissue undergoing autolysis. The opposing theory, strongly supported by Looss ('89) and many others since then, has received most of its evidence from biochemical studies designed to show that typical autolytic processes may occur in the absence of the phagocytes, or at least may be initiated before the appearance of the latter. It has been found, for example, that when the acid production in a cell or a tissue increases to such an extent that it cannot be removed by the buffer mechanism of the blood, autolysis automatically sets in. This fact is of great importance in interpreting the secondary autolytic agents, since it will readily be seen that any factor which tends to either decrease the blood supply of a tissue or to accelerate unduly the accumulation of acid substances will result in the autolysis of the tissue concerned. More detailed discussions concerning the exact operation of these factors are presented by Morse ('18) and Bradley ('22).

Morse ('18), in a study of the factors involved in the atrophy of involuting tadpole organs, has made a biochemical and histological study of the musculature of the atrophying tail. His sections through the latter show that a marked change occurs which he interprets as the first stage of autolysis. This consists of a deposition of fats bearing fatty acids (oleic), which takes place before leucocytes have been attracted to the area. He thus explains autolysis of the tadpole tail as due to an acidosis established in the musculature of the latter, which cannot be caused by the action of leucocytes. The acidosis in this case he interprets according to Bataillon's ('91) explanation of the atrophy of the tail. It will be recalled that the latter author found that the rapid growth of the urostyle hindered the blood supply of the tail.

Mercier ('06) found, however, that complete occlusion of the blood supply was not effected, since carmine granules injected into the dorsal lymph spaces were later found in the atrophying tail muscle of the metamorphosing tadpole. According to Morse, it is not a 'lack of oxygen' resulting from the decreased blood supply which is responsible for the autolysis, but rather the accumulation of CO_2 which results. This tends to raise the acidity of the tissues, since the reduced blood supply is inadequate to remove the acids so formed.

The sections made through the opercular histolytic area, as described in the present paper, correlate strongly with the histological findings of Morse ('18) in his study of atrophying tail muscle. Although the chemistry of the histolyzing integument was not worked out, it was quite apparent that a distinct change in the structure of the integument always preceded the appearance of the lymphocytes. This change, previously described as a congestion of the stratum-compactum fibrils and resulting in their being thrown into more pronounced convolutions, clearly shows that the lymphocytes are of secondary importance as regards their power to initiate histolysis of the integument.

The mechanism employed by the atrophying gills in bringing about histolysis of the opercular integument, while of secondary importance to the problem in hand, is nevertheless an interesting point. It is evident that they are unable to decrease the blood supply of the integument through pressure, since no pressure is exerted by these structures, and even if such were the case, it is doubtful whether this would furnish an adequate explanation, since the fore limb which normally exerts considerable pressure does not induce histolysis. In view of these facts, therefore, and in view of the additional fact that the gills assume histolytic functions only when in a state of atrophy, it seems quite logical to assume the secretion of an histolytic substance of some kind which is produced during a certain period of atrophy by the disintegrating gills. This substance would not necessarily have to be a specific histolyzing hormone, but might be any type of substance capable of deranging the normal acid production of the opercular integument. The fact that only a definite area of opercular integument histolyzes can be accounted for by a consideration of the location of the gills with respect to the former. In fact, the area of histolysis corresponds, roughly, to the position of the gills in the opercular cavity. The area of integument so affected might then be thought of as capable of deranging the metabolism of the integument immediately surrounding it and thus enlarging the histolytic area. That the histolyzing secretion must become weaker in potency or diluted in the integument until it no longer can function must be assumed in this explanation in order to account for the absence of histolysis beyond certain more or less definite boundaries.

Explanations involving the release of an histolyzing hormone into the blood stream or into the opercular cavity would not explain the histolysis of the definite area of opercular integument which occurs. If such were the case, all integument would undergo histolysis, or at least all integument bounding the opercular cavity, since it has been demonstrated that all integument is capable of undergoing histolysis. The mere fact that integument must be transplanted to the opercular region before it will histolyze admits of two possible explanations. Either contact or close proximity of the atrophying gills is essential in order that the histolytic secretion may be transferred to the integument.

It occurred to the writer that since the histolytic effect of gill tissue was only functional during the atrophy of those organs, it might well be that any tissue undergoing a similar atrophy would be capable of inducing integumentary histolysis. Consequently, a series of experiments were performed in which pieces of atrophying tail muscle, taken from metamorphosing tadpoles, just posterior to the hind legs (*M*, text figure B), were placed beneath opercular skin grafts transplanted to the back. In this series eight out of twelve opercular grafts underwent distinct macroscopic and histological signs of histolysis, resulting in the formation of perforations.

The histolysis, however, progressed much more slowly than in the case of the gill-transplant experiments, and the perforations formed were always quite small in size (figs. 25 and 25A).

The fact that atrophying tail muscle is also capable of inducing integumentary histolysis throws new light on the problem. Aside from the evident probability that the histolysis of tail integument is normally brought about by the presence of the underlying atrophying musculature during the absorption of the tail, it suggests the generalization that all atrophying or degenerating tissues in the metamorphosing larvae are capable of initiating histolysis of the integument when brought into contact with the latter. For the purpose of the present investigation, it must be remembered, however, that the *only tissue undergoing such atrophy in the immediate vicinity of the histolytic area, and in close proximity with the integument of the latter, is that of the gills.* It is through their presence alone that histolysis of the opercular skin, as it occurs during metamorphosis, is brought about.

Before closing the discussion of the factors involved in the histolysis of opercular integument, it is necessary to consider the exact functions which might be attributed to the developing fore limbs. The limb-transplantation experiments have shown that the latter are incapable of inducing histolysis of the integument through either the secretion of an histolytic substance or by means of pressure. Their sole function would seem to lie, therefore, in the exertion of pressure against the much-weakened skin of the histolytic area. While it may be possible in some cases, as seems probable when considering the failure of perforations to form in connection with the limb-extirpation experiments on R. clamitans tadpoles, that a certain amount of degeneration of a 'pressure atrophy' type might occur through their presence, it seems safe to conclude that they generally serve only to rupture the histolyzed integument, and so hasten the appearance of an actual perforation. The phenomenon of 'outlining' seems also due to the presence of the fore limb. The outlining generally follows a line in the integument corresponding to the outline of that part of the fore limb lying directly beneath and which is exerting a strong pressure against the former. Since the integument at these points is undoubtedly under the greatest tension, it is probable that a certain amount of pressure atrophy takes place in the skin, visible externally as a thin, white line. The comparatively few cases in which 'outlining' occurs, however, indicate that more than ordinary pressure must be exerted by the fore limb to bring about such a result. In the large majority of cases, therefore, the fore limb functions only by rupturing the histolyzed integument.

The results of the experiments described in this paper, while serving to explain the immediate mechanism involved in the formation of fore-leg perforations during metamorphosis, can also be applied to interpret a more fundamental question pertaining to amphibian metamorphosis in general. This has to do with the mode of action of the thyroid gland in inducing the numerous structural changes that take place during metamorphosis. Briefly stated, the possible modes by which the thyroid can exert action may be of two types, namely, 1) a direct action of the thyroid hormone on the tissue or organ undergoing modification or, 2) an indirect action of the thyroid hormone involving one or more intermediate steps before the organ destined to undergo a metamorphic change is affected. It is evident that a certain amount of direct action must occur during metamorphosis in connection with the thyroid hormone, since the latter must at least be considered the primary activating agent.

In the light of the results of the present investigation, it is evident that many of the structural and physiological changes experienced during metamorphosis are not brought about by a direct action of the thyroid hormone. Thus the thyroid hormone may be thought of as first effecting a certain organ or tissue, which is thereby rendered physiologically or morphologically capable of affecting a second organ or tissue, and so on until, finally, the end result is obtained. The steps involved, for example, in the formation of opercular perforations would seem to be as follows: First, the secretion and release of the thyroid hormone which either directly or indirectly causes an atrophy of the internal gills, and, secondly, the formation of an histolytic substance by the gills so affected, which, when transferred to the opercular integument, initiates histolysis of that tissue. At the same time, the thyroid hormone, directly or indirectly, causes the fore limbs to differentiate rapidly and increase in size, until they function through pressure in rupturing the integument already weakened by the histolytic agent of the atrophying gills. It will be seen, in this connection, that there may be one or more intermediate steps between the secretion of the thyroid hormone and the differentiation of the fore limbs or the atrophy of the gills. Whether or not such steps exist, or whether or not the thyroid acts directly on these particular structures, must remain as problems for future investigation.

The study of the phenomenon of amphibian metamorphosis thus becomes quite complex and difficult, involving not only a careful morphological and histological examination of all tissues undergoing metamorphic changes, but also the determination of the physiological changes and action of all tissues which might even remotely be thought of as influencing the general process or each other in any way. Until this is done, the mechanisms of many of the structural transformations peculiar to certain tissues and organs during metamorphosis, will remain unexplained. Thus, according to this interpretation, the thyroid supplies the initial stimulus for differentiation in anuran transformation, and is but one link, although a very vital one, in a long chain of reactions which it arouses.

GENERAL SUMMARY AND CONCLUSIONS

1. A definite, orderly, process of integumentary histolysis was found to precede the appearance of opercular perforations.

2. The histolysis was shown, histologically, to originate before the appearance of the numerous lymphocytes and other blood cells which are later attracted to the area. Such blood

cells cannot be thought of as initiating opercular histolysis, therefore, but probably function chiefly as phagocytes.

3. Pieces of skin from the side and back of R. clamitans tadpoles were transplanted autoplastically to the opercular histolytic area. In the majority of cases the grafts underwent histolysis during the metamorphosis of the tadpoles.

4. Opercular skin from the histolytic area was transplanted autoplastically to the back and the tadpoles allowed to metamorphose. No histolysis occurred in such transplants.

5. All integument is capable of undergoing histolysis when transplanted to the region of the operculum which normally undergoes histolysis. The opercular integument of the frog tadpole is therefore not locally specific as regards its ability to histolyze during metamorphosis.

6. The opercular skin, which normally undergoes histolysis during metamorphosis, is not self-differentiating as to its ability to histolyze when transplanted autoplastically or homoplastically to other parts of the body.

7. The right fore limbs of R. clamitans, R. pipiens, and R. sylvatica tadpoles were extirpated at various stages of development, and the animals allowed to metamorphose. Histolysis of opercular integument occurred in all three series, resulting in the formation of perforations in all except the R. clamitans series.

8. The presence of the developing fore limb is therefore not essential for the histolysis of opercular integument as it occurs during metamorphosis. The fore limb in R. clamitans tadpoles probably functions mechanically in hastening the formation of a perforation.

9. Fore-limb buds of R. palustris tadpoles were transplanted homoplastically beneath the integument of the back, and the animals artifically metamorphosed. Only three perforations out of twenty-five possible cases formed. No histological signs of histolysis were found connected with such perforations. The rupturing of the integument was due solely to pressure.

10. Glass beads were transplanted beneath the side integument of tadpoles of R. clamitans and R. pipiens. Out of twenty cases, sixteen perforations resulted, due to the exertion of pressure by the glass beads. No histological signs of histolysis were found in connection with the formation of these perforations.

11. The developing fore limb of metamorphosing tadpoles is incapable, therefore, of initiating histolysis of opercular integument either through pressure or through the secretion of an enzymatic substance. In certain cases, however, the fore limb may produce a small amount of so-called 'pressure atrophy.'

12. The developing fore limb functions during metamorphosis simply by rupturing the histolyzed opercular integument through pressure, and thereby hastens the appearance of an actual perforation.

13. Pieces of gill tissue in various stages of atrophy and degeneration were transplanted homoplastically from metamorphosing tadpoles of R. palustris and R. pipiens, either under the normal integument of the back or under opercular skin grafts placed on the back. Histolysis of the integument rapidly followed and resulted in large perforations.

14. The atrophying gills of metamorphosing anuran larvae are, therefore, the immediate histolytic agents concerned in the histolysis of opercular integument as it occurs in metamorphosing anuran larvae.

15. The atrophying gills probably initiate opercular integumentary histolysis through the secretion of an histolytic substance which is transferred to the integument.

16. Pieces of atrophying tail muscle were similarly transplanted homoplastically beneath opercular skin grafts transplanted to the back. Histolysis of the grafts occurred resulting in the formation of small perforations. The rate of histolysis was much slower than when atrophying gill tissue was used.

17. It seems probable, therefore, that any atrophying tissue in close proximity with the opercular integument is

able to initiate histolysis of the latter during metamorphosis. The only atrophying tissue in the immediate vicinity of the opercular histolytic area, however, is that of the gills.

18. The thyroid hormone acts indirectly in producing the opercular fore-limb perforations of anuran larvae during metamorphosis.

19. The thyroid hormone is in many cases simply the primary activating influence of metamorphosis and does not act directly in bringing about transformation, atrophy, or development of certain structures during the process of metamorphosis.

20. We must regard the thyroid, therefore, as simply the primary activating agent in what may often prove to be a long series of intermediate and dependent factors destined eventually to bring about certain definite metamorphic transformations.

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PLATE 1

EXPLANATION OF FIGURES

1 to 5 Sections through the histolytic area of the operculum of a tadpole, showing the successive steps of histolysis leading up to the formation of the foreleg perforation (R. clamitans). *E,* epidermis; *SS,* stratum spongiosum; *SC,* stratum compactum; *SBS,* subcutaneous connective tissue; *EL,* epithelial lining; *P,* pigment masses; *X,* region of histolysis; *L,* lymphocytes; *BV,* blood vessel. × 90.

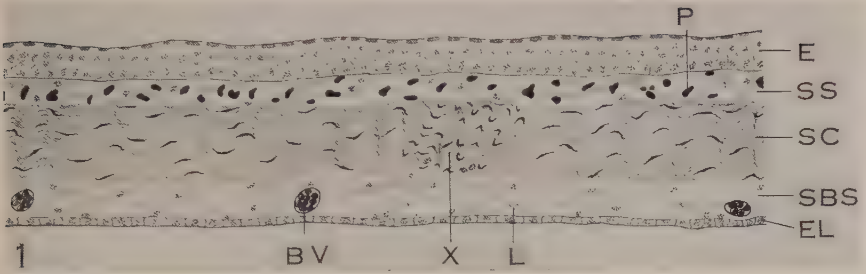
1 First histological sign of histolysis. Fibrils of stratum compactum are thrown into more pronounced convolutions.

2 Stratum compactum and stratum spongiosum are dissociated. Blood cells, chiefly lymphocytes, begin to invade the histolytic area.

3 The histolysis is well under way resulting in a 'thinning' of the skin. Lymphocytes are very numerous.

4 Thinning more pronounced. The subcutaneous connective tissue is absent.

5 Only a single layer of epidermal cells (the cuticle) and a few fibrils of the stratum compactum remain. At this stage the rapidly growing fore limb, which is exerting considerable pressure, is able to rupture this layer and emerge through the perforation so formed.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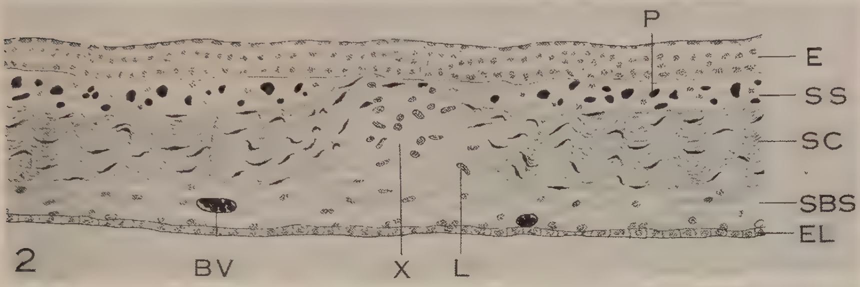
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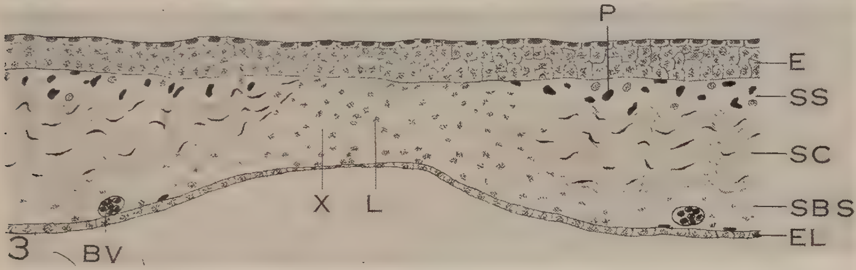
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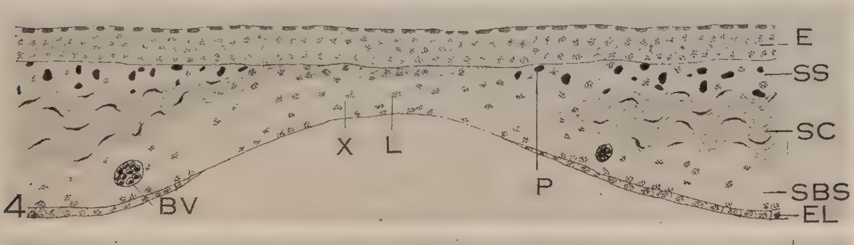
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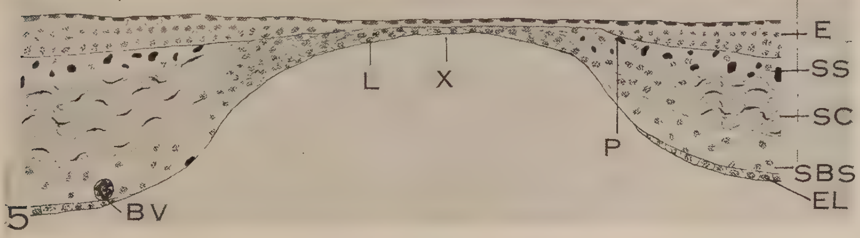
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PLATE 2

EXPLANATION OF FIGURES

6 to 9 Sections through the edge or 'shelf' of the opercular perforation, showing its gradual histolysis and fusion with the newly formed skin from the fore limb (R. clamitans). *E*, edge of perforation; *FS*, fore-leg skin; *L*, lymphocytes; *C*, connective-tissue-forming elements. The various layers of the integument correspond to those labeled in plate 1. × 70.

6 Entire central portion of perforation edge composed of dissociated fibrils of stratum compactum and of other dermal elements, heavily invaded by lymphocytes. The stratum compactum of the fore-leg skin has not, as yet, made connections with that of the opercular skin. Connective-tissue-forming elements may be seen, however, in the process of connecting the two layers.

7 Perforation edge considerably atrophied.

8 Perforation edge no longer projects out, due to a further atrophy.

9 Perforation edge indicated only by a notch. All layers of the fore-leg and opercular integuments are by now practically continuous.

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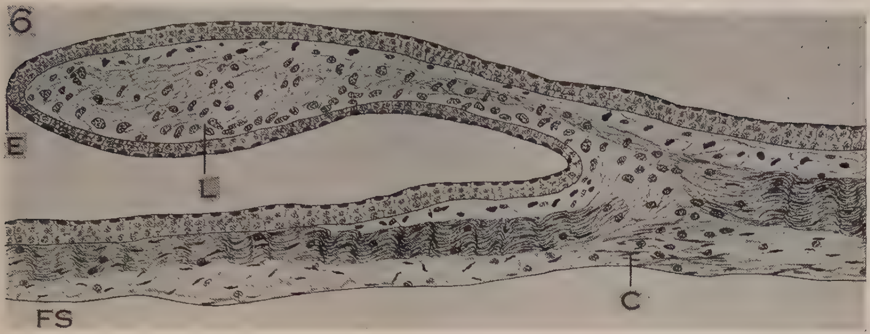

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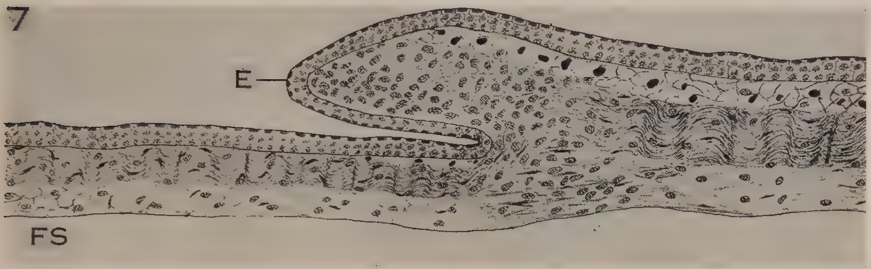

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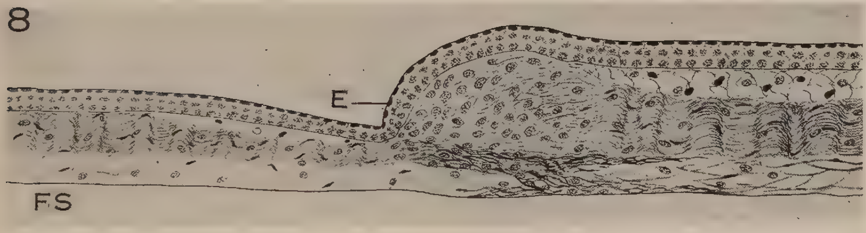

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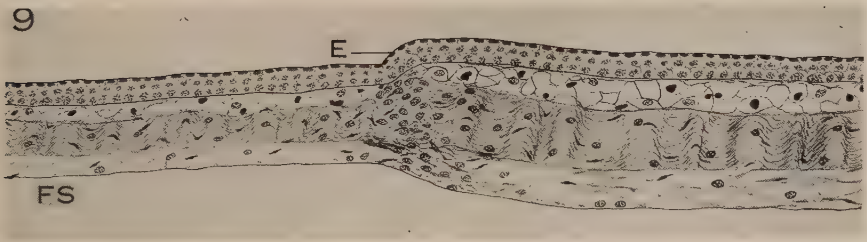

PLATE 3

EXPLANATION OF FIGURES

10 to 14 Sections through the integument containing perforations which occurred in connection with the various types of operations performed. The various layers of the integument correspond to those labeled in plate 1.

10 Back-graft perforation several days after its formation. The graft has undergone a typical process of histolysis and is heavily invaded by lymphocytes. *BG*, integument of back graft; *FS*, integument from the fore-limb; *E*, edge of perforation. (Case SC 31, R. clamitans.) × 70.

11 Fore-limb extirpation perforation several days after its formation. The histolysis is identical with that found when the fore leg is left intact. *E*, edge of perforation. (Case EP 13, R. pipiens.) × 90.

12 Limb-bud transplant perforation shortly after formation. The perforation is clearly the result of a rupturing of the skin by the fore leg through pressure. No histolysis is present. *P*, perforation. (Case LT 19, R. palustris.) × 400.

13 Bead-transplant perforation two days after its formation. The perforation is evidently the result of pressure exerted by the bead, since no distinct signs of histolysis are present. *E*, edge of perforation; *L*, lymphocytes. (Case BT 2, R. clamitans.) × 105.

14 Gill-transplant perforation shortly after its formation. The histolysis of the integument is so pronounced that liquefaction of the stratum compactum of the opercular graft has occurred at *A*. *E*, edge of perforation; *G*, atrophying gill-transplant filling perforation; *L*, lymphocytes; *Y*, portions of stratum compactum which have become separated from the main layer. (Case GP 10, R. pipiens.) × 100.

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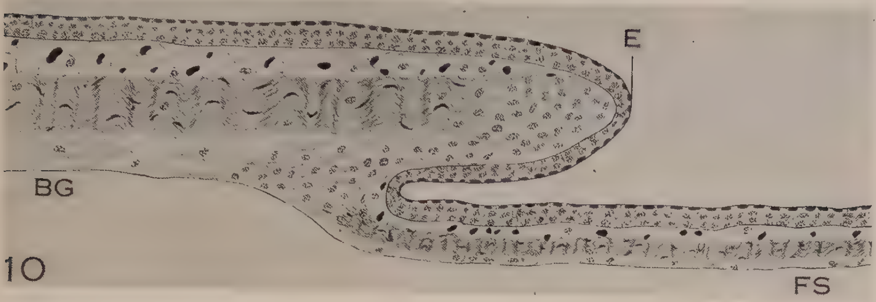

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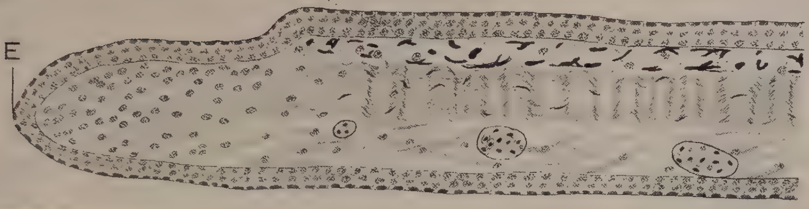

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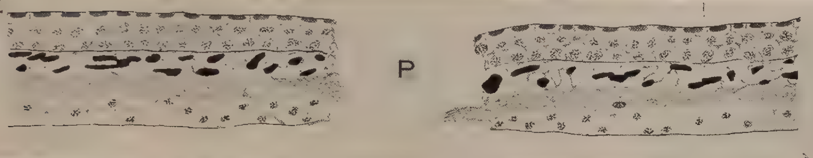

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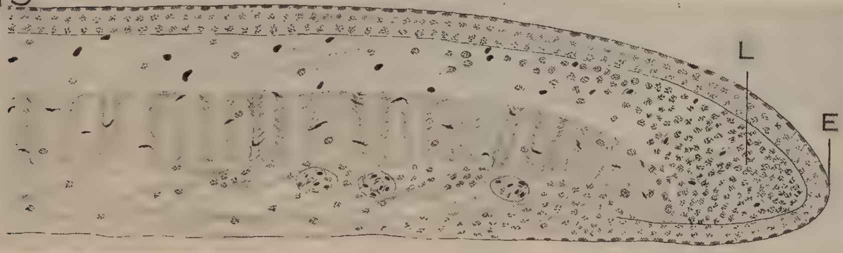

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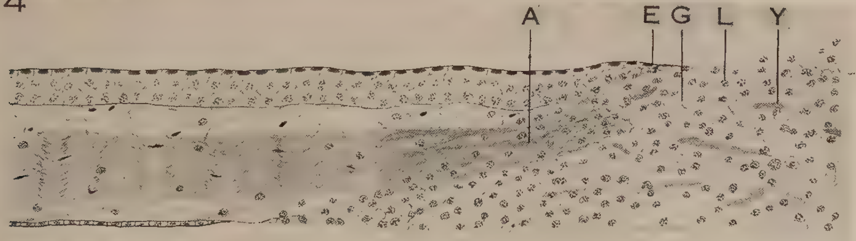


PLATE 4

EXPLANATION OF FIGURES

15 Macroscopic histolysis of a side graft during metamorphosis, which had previously been transplanted to the histolytic area. *P*, perforation; *SG*, side-skin graft transplanted to the opercular region. (Case SC 11, R. clamitans.) × 2.

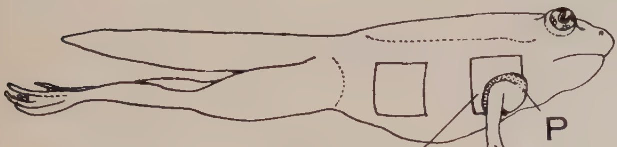
16 Formation of a small perforation which occurred in the absence of the fore limb during metamorphosis. *P*, perforation. (Case ES 10, R. sylvatica.) × 2.

17 Formation of a perforation due to pressure of transplanted glass bead. *P*, perforation with bead showing beneath. (Case BT 11, R. pipiens.) × 1⅓.

18 Formation of a large perforation, which occurred in the absence of the fore limb during metamorphosis. The atrophying gills are visible and partly projecting through the perforation. (Case EP 1, R. pipiens.) *P*, perforation. × 2.

19 Outlining in histolytic area which occurred in the absence of the fore limb during metamorphosis. Very slight, if any, signs of macroscopic histolysis present. *OA*, outlined area. (Case EC 3, R. clamitans.) × 1⅕.

20 Formation of two perforations due to pressure exerted by glass-bead transplant. *P*, perforations. (Case BT 1, R. clamitans.) × 1⅓.



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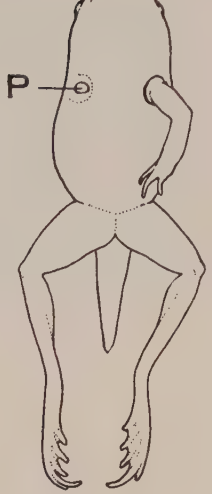

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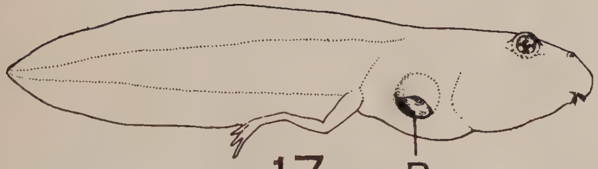

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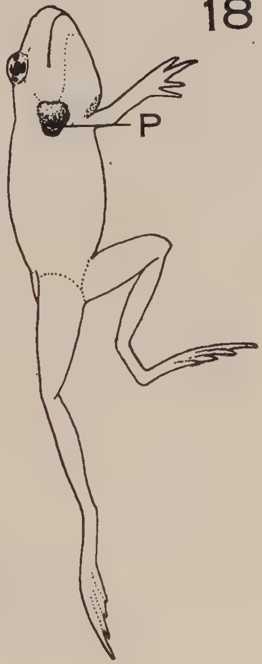

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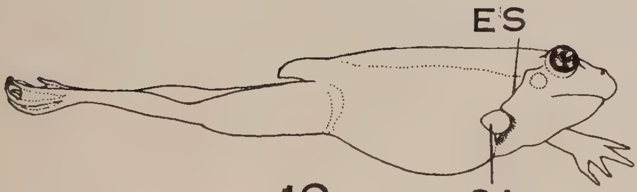

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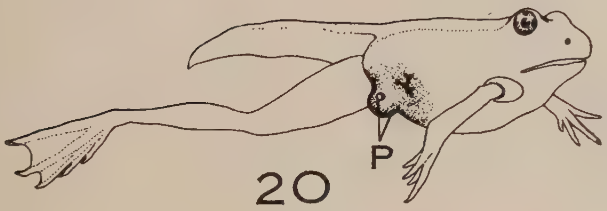

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PLATE 5

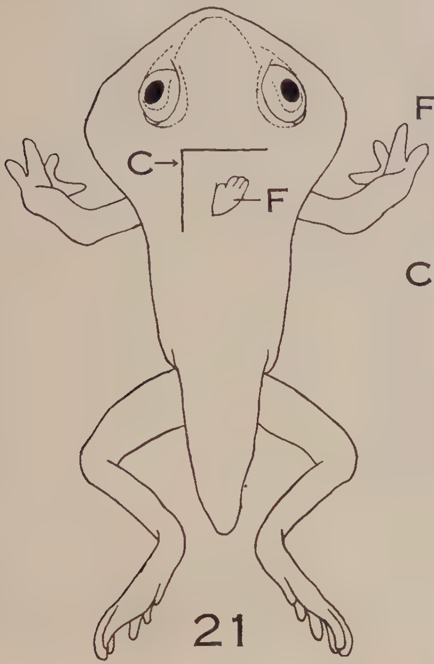
EXPLANATION OF FIGURES

21 One of the three limb-bud transplant cases in which a perforation occurred. (Case LT 19, R. palustris.) × 7.

21A Binocular sketch of perforation area of case LT 19, showing fore limb which had differentiated and succeeded in rupturing the integument of the back. *F*, fore limb protruding through the perforation; *C*, cuts previously made in laying back the integument for the transplantation of the fore-limb bud. × 13.

22 Appearance of macroscopic signs of histolysis in the absence of a fore limb during metamorphosis. *T*, visible 'thinning' of histolytic area; *P*, small perforation developed in histolytic area. (Case EP 19, R. pipiens.) × $2\frac{2}{3}$.

23 Bead-transplant perforation, the result of pressure exerted by the glass-bead transplant. The animal was still typically tadpole-like in appearance. (Case BT 2, R. clamitans.) *P*, perforation. × $1\frac{2}{3}$.



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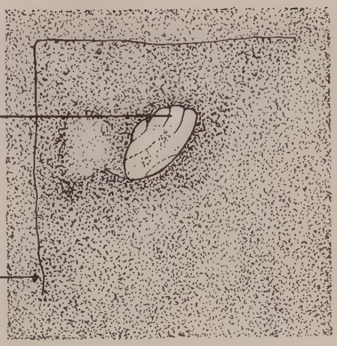

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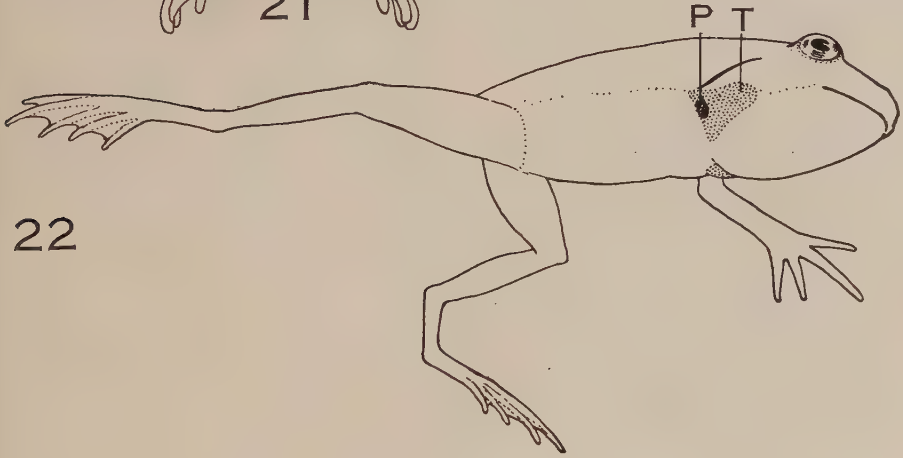

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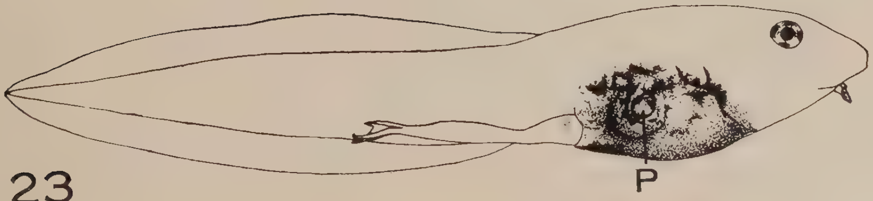

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PLATE 6

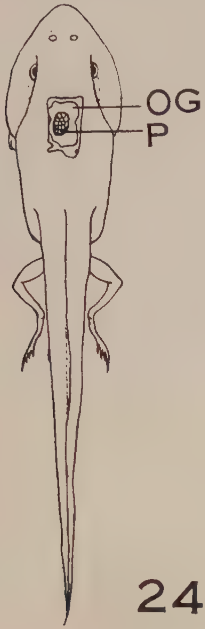
EXPLANATION OF FIGURES

24 Gill transplant perforation formed in the opercular skin graft previously transplanted to the back. (Case GP 9, R. pipiens.) × 1⅓.

24A Binocular sketch of opercular skin graft and host skin of case GP 9, R. pipiens. *OG*, opercular skin graft; *P*, perforation; *G*, atrophying gill transplant beneath skin graft and filling perforation. × 11.

25 Tail-muscle perforation formed in the opercular skin graft previously transplanted to the back. (Case MT 4, R. palustris.) × 5.

25A Binocular sketch of opercular skin graft and host skin of case MT 4, R. palustris. *OG*, opercular skin graft; *P*, perforation showing atrophying tail muscle transplant beneath skin graft. × 18.



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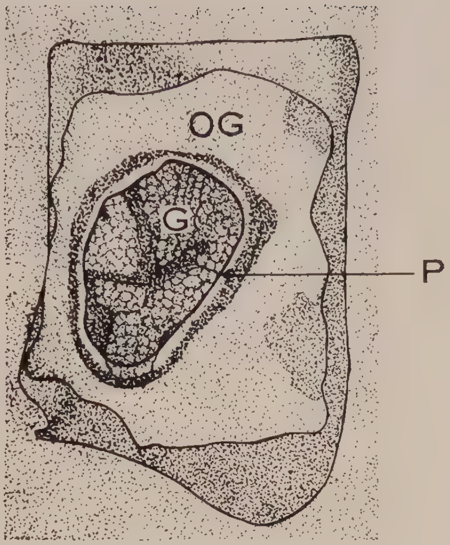

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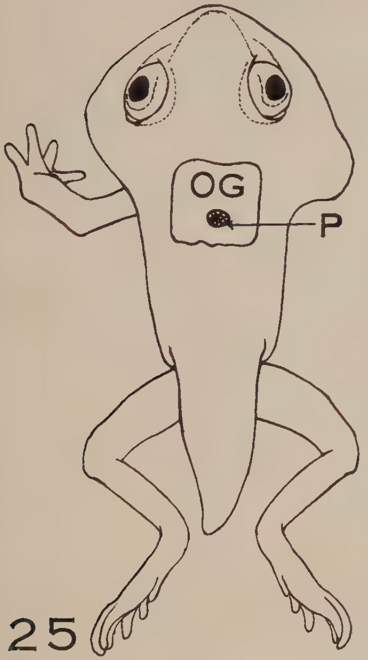

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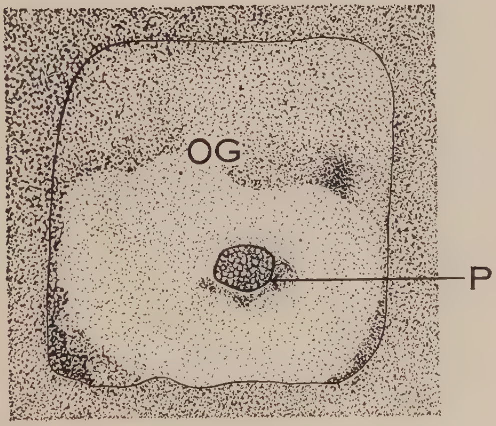

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STUDIES ON AMPHIBIAN METAMORPHOSIS

II. THE OXYGEN CONSUMPTION OF TADPOLES UNDERGOING PRECOCIOUS METAMORPHOSIS FOLLOWING TREATMENT WITH THYROID AND DI-IODOTYROSINE

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FIVE FIGURES

CONTENTS

Introduction 69
Materials and methods 72
Di-iodotyrosine metamorphosis 75
  A. Stages of metamorphosis tested 75
  B. Di-iodotyrosine and natural metamorphosis 77
Weight reduction during metamorphosis 79
Oxygen consumption of stages tested 82
Effect of etherization on oxygen consumption 85
Discussion 86
Summary and conclusions 89
Literature cited 90

INTRODUCTION

The nature of certain of the secretory products of the thyroid gland responsible for inducing metamorphosis has been determined since Gudernatsch's ('12, '14) original discovery of the rôle of the thyroid in metamorphosis. Swingle ('19) and Swingle, Helff, and Zwemer ('24) have shown that it is the organically combined iodine in the thyroid molecule which is really directly concerned in the metamorphic process. Numerous injection and feeding experiments of iodized compounds, such as iodotyrosine, iodocasein, iodoserumalbumin, and iodoserumglobulin, have since shown that metamorphosis may be rapidly induced even in thyrodectomized individuals, showing that it is not necessary for the thyroid gland to absorb and recombine the iodine, providing it is in the right organic combination when administered.

Although many problems relating to the release of the thyroid colloid, its relationship to the functioning and secretion of other glands, notably the pituitary, and the exact method by which it operates to bring about the numerous metamorphic changes that occur, remain to be solved, the fact is evident that a striking change in the metabolism of the organism must occur prior to the onset of metamorphosis when the secretion is released into the blood stream. The profound katabolic processes at work in the destruction of the tail and the equally striking anabolic phase operating in the development of the limbs are strong evidences, at least, of marked metabolic changes.

A consideration of the many local changes in metabolism which take place would certainly lead one to suspect a change in the total metabolism of the organism. Such being the case, the respiratory rate of involuting larvae should be strongly affected and in the opinion of the writer should show a marked increase, roughly proportional to the degree and speed of the morphological transformations in progess. Experiments designed, therefore, to measure the changes in CO_2 production or O_2 consumption during metamorphosis are of interest in that CO_2 production and O_2 consumption are generally conceded to give a rough estimate, at least, of the degree of total metabolism.

As far as the writer is aware, but two workers have directly concerned themselves with this mode of attack. Groebbels ('22) determined the oxygen consumption of R. temporaria larvae, some of which were undergoing a natural metamorphosis and some in which metamorphosis had been induced by thyroid treatment. Those undergoing natural metamorphosis were found to consume more oxygen per gram weight but less oxygen per animal when compared with larvae showing no signs of metamorphosis. The tadpoles metamorphosed by thyroid treatment were found to consume not only more oxygen per gram weight, but also more oxygen per animal when compared with larvae not undergoing metamorphosis. This would indicate that the metabolic rate of artificially metamorphosing R. temporaria larvae increases during transformation. The following year, Abelin and Scheinfinkel ('23) published the results of their work on R. esculenta and axolotl (Amblystoma mexicanum), in which the CO_2 production of artificially metamorphosing larvae was measured. The CO_2 production of both these forms slightly increased during the first few days following thyroid feeding. As the larvae of R. esculenta metamorphosed, however, a decrease in CO_2 production was recorded amounting to from 50 to 70 per cent less at the end of metamorphosis as compared with larvae exhibiting no signs of metamorphosis. Similar experiments made on di-iodotyrosine and di-iodotyramin metamorphosing R. esculenta larvae showed that half-metamorphosed larvae were liberating from 40 to 70 per cent less CO_2 than tadpoles not undergoing metamorphosis. These results, although seemingly diametrically opposed to those found by Groebbels, can be explained to a certain extent on the ground that the CO_2 production of the larvae tested was not calculated per gram weight, but on the animal as a whole. Further reference to this apparent dissimilarity in results will be taken up more fully in discussing the results of the present paper.

The present investigation was completed during the summer of 1923 and an abstract of the results published in November of the same year (Helff, '23). The work involves data collected in connection with over five hundred oxygen-consumption tests made on the larvae of R. pipiens. The individual testing of larvae in practically every important stage of metamorphosis, together with their weights and related data, was thought to be of use in arriving at an adequate interpretation of the problem under investigation. Preliminary tests had convinced the writer that, due to the amount of variation existing between like individuals of the same metamorphic stage, conclusions could be reached only through the examination of a large number of specimens and of a graded series of metamorphic stages.

The writer is indebted to Dr. W. W. Swingle of this laboratory for suggesting the problem and for his kindly interest throughout the course of the work.

MATERIALS AND METHODS

The species selected for the experiment in hand was that of R. pipiens. The selection was based on the fact that the larvae of this species were obtainable in great abundance from a small pool near Foxon, Connecticut. Furthermore, larvae of this species had proved to lend themselves more favorably to the exigencies of artificial metamorphosis than had R. catesbeiana and R. clamitans larvae. The larvae were collected during the months of June, July, August, and September, 1923.

The individuals selected for use in the tests ranged from 46 to 55 mm. in length and were normal tadpoles in every respect. The hind limbs of such specimens varied from 2 to 10 mm. in length. The average body length was about 50 mm. and the average hind limb length 5 mm. When brought to the laboratory, the larvae were first sorted as regards their apparent viability and the more vigorous appearing specimens isolated in small battery jar aquaria. All specimens so isolated were maintained for a period of three to four days under constant food and temperature conditions, in order that those which were not adequately adapted to a laboratory environment could be eliminated before being used in the tests. This precaution was very desirable, since it greatly reduced the mortality rate, which, at best, is always high in a series of artificially metamorphosing tadpoles. The temperature of the water in the individual aquaria was maintained at 22° ± 1°C., while the food consisted of a daily feeding of Spirogyra. The selection of a suitable diet, prior to the onset of artificial metamorphosis, is a matter of considerable importance. Preliminary experiments had shown that a liberal feeding of Spirogyra once a day for three days preceding the administration of the metamorphic agent surpassed by far a series of other foods, such as casein, desiccated thymus, powdered cracker, chopped worms or liver, etc. Spirogyra-fed larvae invariably fatten within three days and are very active and healthy and able to withstand artificial metamorphosis much better than if fed on other foods or not at all.

The oxygen-consumption tests were made by the Winkler method for determining the amount of dissolved oxygen in water. This method, although sharing to a certain extent the experimental errors common to all methods for determining the amount of dissolved oxygen in water, is, nevertheless, generally conceded to be the most accurate. It is especially valuable and reliable where comparative work is concerned and where considerable differences in oxygen consumption are shown to exist. It shares, however, the common inaccuracy of all such methods where an exact quantitative determination of the oxygen consumption of a single specimen is desired. For this reason it becomes more and more accurate where a considerable number of like individuals are tested and an average taken to represent the oxygen consumption of such specimens.

The writer has found that certain precautions will greatly facilitate the accuracy of the tests. These include a carefully standardized burette with graduations to the one-hundredth cubic centimeter and a constant burette reading light. In addition to the usual care in standardizing the various chemical solutions used and the preparation of testing water rendered as nearly constant as possible, as regards temperature and oxygen content, it was found that the use of an N/200 sodium thiosulphate solution for titrating was exceedingly valuable in lending accuracy, as compared with the N/100 solution usually employed.

The elimination of as many factors as possible which might influence in any way the oxygen consumption of the larvae during tests is a matter of first importance. In this connection an effort was always made to eliminate those individuals from the tests that were either abnormally active or sluggish as compared with the average specimen. Secondly, as previously indicated, the age, size, and metamorphic stage of individuals employed were maintained as nearly constant as possible, considering the number of specimens tested. A third precaution consisted in the starvation of all specimens for at least twenty-four hours preceding the initial test. This removed any variation which might be due to the peristaltic movements of the digestive tract in the digestion of food, such movements being quite definitely known to increase oxygen consumption.

The stage 1 or 'normal tadpoles' tested were usually quite active and were therefore always anaesthetized in order to eliminate errors in the oxygen consumption due to muscular activity. The anaesthetic used was a 2 per cent aqueous solution of ether. The tadpoles were immersed in this solution long enough so that they remained anaesthetized for from five to ten minutes. Larvae so anaesthetized remained motionless during the following two-hour testing period, providing the flasks were not moved or agitated in any way. Tadpoles in various stages of metamorphosis required no anaesthetic, since they invariably remained quiet during the testing period.

The natural metamorphosis of R. pipiens larvae, although desirable for the experiment in hand, is unfortunately restricted to a period of two or three weeks in August, which fact made the employment of an artificial metamorphic agent imperative. Preliminary series of stock animals were subjected to several of the methods known to induce artificial metamorphosis, which included feeding of desiccated thyroid or thyroxin tablets, intraperitoneal injections of extracts of frog or cat thyroid glands, and transplantation of frog, axolotl, or cat thyroid tissue, intraperitoneally. Although some of these methods were fairly successful, they all shared the common fault of not producing a gradual and complete metamorphosis. In fact, it not uncommonly happened that only a certain percentage of the animals so treated responded to the agent, the others remaining apparently unaffected, while those which responded to the agent became edematous and died.

It was finally found that the administration of desiccated thyroid (Parke, Davis & Co., containing 0.20 per cent iodine by weight), followed by immersion in a solution of di-iodotyrosine, produced the desired results. The animals to be metamorphosed were first fed the desiccated thyroid for two days, which was sufficient to give the initial stimulus for transformation. They were then placed in fresh water to which a small amount of finely pulverized di-iodotyrosine had been added. The di-iodotyrosine solution produces a steady and constant stimulus, since the compound is taken in not only via the digestive tract, but also directly through the integument. This latter fact undoubtedly accounts for the steady and complete metamorphosis attained, since the cessation of the feeding habit typical of metamorphosing larvae does not prevent the absorption of the di-iodotyrosine. It was further found that the larvae could be left in the original di-iodotyrosine solution throughout their entire transformation, the solution seldom becoming foul and apparently retaining sufficient strength to induce complete metamorphosis. Di-iodotyrosine metamorphosis also possesses the additional advantage of not introducing errors due to digestive phenomena, which is generally the case with feeding methods. The type of metamorphosis produced by di-iodotyrosine differs somewhat from the natural metamorphosis of R. pipiens larvae and will be discussed below.

DI-IODOTYROSINE METAMORPHOSIS

A. Stages of metamorphosis tested

The employment of di-iodotyrosine made possible the selection of as many stages of metamorphosis as seemed desirable and necessary for the purpose of the experiment. Consequently, nine stages were arbitrarily selected as shown in figure 1. A brief description here of each stage so selected may serve to clarify the later discussion of their oxygen consumption.

Stage 1. Stage 1 larvae, as previously described, were those selected for the initial tests and were typical tadpoles in every respect, with hind limbs averaging 5 mm. in length. Other than this slight differentiation of the hind limbs, there were no visible external signs of metamorphosis and presumably no internal changes.

Stage 1 a. Tadpoles in this stage gave evidence of first signs of metamorphosis, such as reduced activity and an increased differentiation and growth of the hind limbs. The tail was also slightly atrophied dorsoventrally in this stage. This stage was not tested, as little or no internal changes had as yet occurred.

Stage 2. Stage 2 tadpoles were distinctive in two regards: 1) the atrophy of the tail anteroposteriorly and, 2) the loss of the typically rounded contour of the body due to a constriction of the integument resulting from intestinal atrophy and reorganization.

Stage 3. This stage represented a still further atrophy of the tail in which the dorsal and ventral fins were reduced to mere ridges.

Stage 4. Tadpoles in this stage gave evidence of the first signs of frog-like structures, such as mouth changes and typical frog-like hind limbs. The fore limbs at this stage were quite well developed within the opercular cavity and were slightly bulging the integument covering the latter. The tail had considerably shortened and lost all vestiges of its dorsal and ventral finny portions.

Stage 5. The fore limbs in this stage produced a very strong bulging of the opercular integument, while the tail was quite short and wholly black in color. The mouth was nearing the end of its transformation from the tadpole to the frog type.

Stage 6. The chief characteristic of this stage was the protrusion of the left fore limb elbow. The integument had begun to change its pigmentation and assume the lighter frog-like coloration.

Stage 7. Stage 7 was characterized by the complete eruption of the left fore limb, while the tail was reduced to a short and barely movable black stump.

Stage 8. Stage 8 represented the last stage tested, being characterized by a further diminution of the tail stump, an increase in the left fore-limb growth and development of the typical frog mouth and pigmentation. The animal in many cases was now able to assume a frog-like sitting posture.

The time required for a tadpole to pass from stage 1 to stage 8 varied between nine and twelve days. This variation must be attributed to individual physiological differences, since the strength of the di-iodotyrosine solution was quite constant as were the other environmental factors. The eight successive stages were reached, on the average, within three, five, six, seven, eight, nine, ten, and eleven days after the immersion of stage 1 tadpoles in the di-iodotyrosine solution. The remarkably constant metamorphosis obtained was of considerable help in planning the testing periods of the large number of larvae undergoing transformation. The attempt was made at first to test the larvae during every stage of metamorphosis, but the excessive handling and weighing of the specimens proved to be fatal, practically all dying before the later stages were reached. It was finally found that by using a larger number of animals and testing at only every other stage sufficient data on all stages could be obtained.

B. Di-iodotyrosine and natural metamorphosis

Artificially induced metamorphosis is at the best but a rough approximation to natural metamorphosis, as has long been recognized by practically everyone using artificial methods. The use of di-iodotyrosine as a metamorphic agent, however, is comparatively recent and has not been employed to any great extent, due, no doubt, to the fact that it is not a commercial product and must be prepared by the individual worker. It would seem of importance to discuss at this time, therefore, the points in which metamorphosis induced by this agent differs from the natural metamorphosis of R. pipiens larvae.

Perhaps the most noticeable, distinctive characteristic of di-iodotyrosine metamorphosis is the peculiar wedge-shaped form which the larvae assume during late stages of metamorphosis. This condition may be likened somewhat to the 'pigmy frogs' of Gudernatsch. Of hardly secondary importance is the deep black coloration which the larvae assume after several days' immersion in the solution, and this pigmentation becomes more pronounced as metamorphosis progresses. Accompanying both of these changes is a general emaciation of the body similar to that produced by continued desiccated thyroid feeding, which greatly weakens the larvae and, with the exception of a few cases, generally results in their death shortly after stage 8 is reached. It is possible, however, to produce complete metamorphosis in larvae by using very dilute solutions of di-iodotyrosine, in which case the rate of metamorphosis is greatly retarded, so that a period of three weeks is necessary for complete transformation.

The comparison of a series of di-iodotyrosine metamorphosed larvae with a series of larvae undergoing natural metamorphosis is of interest. The writer was fortunate in obtaining a series of larvae from the same stock as used in the tests, which were undergoing metamorphosis naturally. Representative members of this series are pictured in figure 2, while figure 3 shows a dorsal view of the first five stages of the di-iodotyrosine series pictured in figure 1. Figure 4 is a lateral view of the same five stages. The specimens in figure 2 are labeled with the stage number corresponding to the same stage in the di-iodotyrosine series, using the limb development as a criterion.

A close examination of the two series as pictured in figures 1, 2, 3, and 4 will reveal certain pronounced differences as regards the degree of correlation of atrophying and differentiating structures to the stage of metamorphosis. The atrophy of the tail is especially definite in this connection. In the di-iodotyrosine series it will be noted that it is one of the first structures to undergo a change, and, in fact, it is reduced to a small blackened stump by the time stage 7 is reached, at which time the left fore limb has just appeared. If we compare this situation to stage 7 of figure 2, we find that, although the left fore limb has emerged, the tail is but half atrophied dorsoventrally and only very slightly reduced in length. This would indicate that the tail tissues of the di-iodotyrosine larvae are unduly accelerated in their degeneration as compared to the atrophy which occurs during normal metamorphosis. In a like manner, it can be pointed out that while the limbs of the artificially metamorphosing larvae grow very rapidly and attain the same lengths, as is the case of the limbs of naturally metamorphosing larvae, they do not acquire the thickness or strength of the latter. Furthermore, the right fore limbs of the former type are generally greatly retarded and, in fact, seldom appear, since the larvae invariably die within four days following the attainment of stage 8. In the natural metamorphosis of R. pipiens larvae the right fore limb generally appears within a day following the eruption of the left fore limb.

The various differences exhibited in di-iodotyrosine larvae as compared with naturally transforming specimens show clearly, therefore, that normal correlation of development and degeneration of organs and parts is greatly altered and quite specific for the type of metamorphic agent employed. If we assume that the same lack of normal correlation exists with the internal organs, we are not surprised at the rather high mortality encountered during artificial metamorphosis.

WEIGHT REDUCTION DURING METAMORPHOSIS

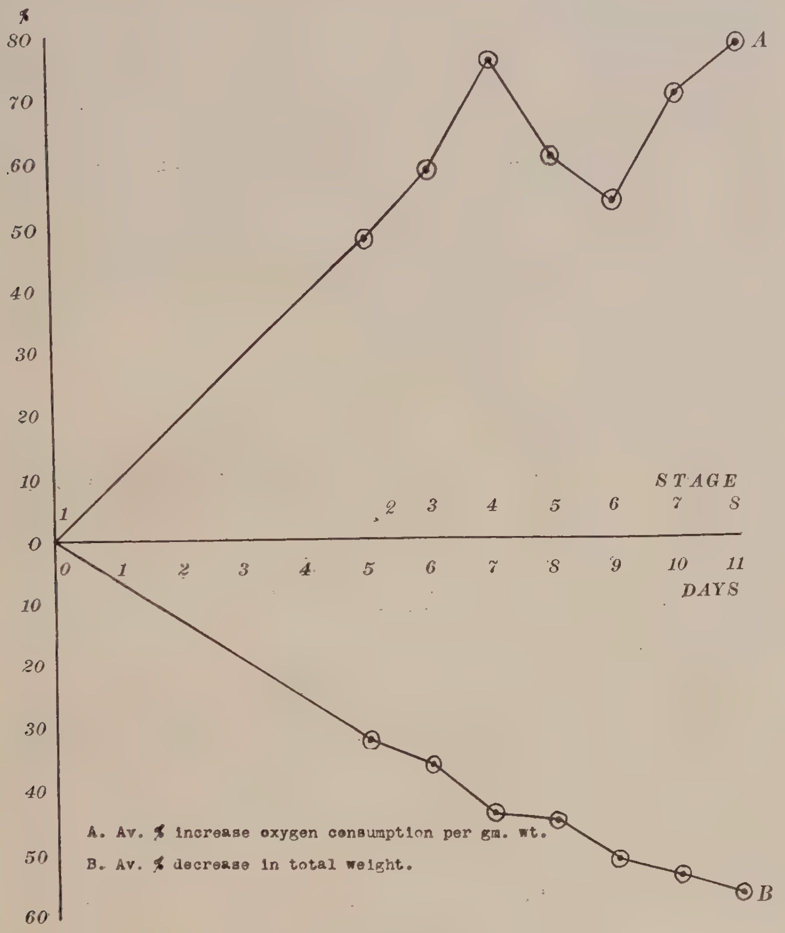
The determination of the metabolic rate of the various stages of metamorphosing larvae required an accurate weighing of the larvae to the milligram at the time the tests were made. The specimens were first dried of superfluous water by placing them on a damp cloth and then weighed in small dishes of water which had previously been balanced on the scales. In this way variable losses due to the drying off of the animals were reduced to a minimum and they were able to survive several weighings without injury. It may be mentioned in this respect that metamorphosing larvae react very delicately to excessive handling and adverse environmental conditions; dry weighing, for example, often proves fatal in certain stages of metamorphosis if not carried out with great rapidity.

TABLE 1

Average weight in grams

NUMBER TESTED	STAGE								AVERAGE DECREASE
	1	2	3	4	5	6	7	8	
32	1.394	.942							.452
19	1.451		.922						.529
33	1.409			.793					.616
36	1.398				.765				.633
16	1.375					.662			.713
27	1.400						.643		.757
10	1.415							.605	.810

The determination of the average weight of larvae tested for each metamorphic stage (table 1 and text figure A) is of interest in considering the changes in body weight that occur during metamorphosis. Stage 2 larvae show the greatest decrease (32 per cent) in body weight of any stage weighed, as compared to stage 1 larvae. From stage 2 on there is a gradual and progressive decrease amounting to 36, 44, 45, 52, 54, and 57 per cent, respectively, for the remaining six stages. It seems quite probable that a considerable further decrease would have been found to occur had more advanced stages than stage 8 been obtainable. The marked reduction in stage 2 over stage 1 larvae can be partially accounted for by the fact that the intestinal tract is by this time entirely evacuated. Stage 1 larvae when tested, although previously starved for at least twenty-four hours, still possess a considerable amount of partially digested food and feces, which is not entirely eliminated until several days afterward. In addition to this factor, there is undoubtedly a considerable decrease in weight due to a loss of water by the various tissues (Yung, '85).



Text figure A

The relatively slight decrease found in the succeeding stages can be explained on the weight changes of the various organs and parts undergoing transformation. This change is necessarily a slight one, in spite of the profound atrophic and regenerative changes that occur, since metamorphosis involves an economical process in which the degenerating tissue elements of one organ are built up into the developing structure of another. Thus, little change in the total weight of the transforming larva is produced. The fact that a certain decrease does progressively occur would seem to indicate, however, that a certain amount of the degenerating substances cannot be assimilated in the building of new structures and is therefore eliminated, presumably in the form of gases. The general and typical emaciation produced by di-iodotyrosine metamorphosis would also lead one to expect that weight reduction is greater in animals so transformed than in those undergoing natural metamorphosis.

OXYGEN CONSUMPTION OF STAGES TESTED

The actual testing of the transforming larvae involved a careful daily inspection of the specimens and the selection of those which had reached the desired stage. The testing was never carried on with groups of larvae in the same stage, but always individually, in order that the degree of variation existing among members of the same stage could be determined. The individual testing of the larvae was furthermore desirable in that occasional defective specimens exhibiting extremely low respiration could be eliminated. Such specimens usually died within a day after the test. The calculation of the oxygen consumption was always based on the amount absorbed per gram weight, in order that a measure of the metabolic rate could be reached. The consumptions per gram weight of all specimens tested were then averaged and the result regarded as the true measure of the metabolic rate of that particular stage of metamorphosis.

Over 300 stage 1 (normal) larvae were tested individually. Many of these, however, were not used for further testing, since they succumbed under the influence of the metamorphic agents before more advanced stages were reached. The average oxygen consumption for the entire number of stage 1 larvae tested was .172 ± .008 cc. oxygen per gram weight. The individual variation was from 0.152 to 0.208, while the vast majority fell between 0.165 cc. and 0.180 cc.

The evacuation of a certain amount of feces by stage 1 larvae during the period in which they were confined to the testing flasks suggested that the feces might be combining with the iodine set free during the test and so introducing serious errors. Since the amount of feces thrown off varied considerably between individuals of stage 1 during the testing period, it seemed quite probable that the extreme variations in oxygen consumption calculated might be due to this cause. Accordingly, tests were devised to determine whether or not the feces actually combined with the iodine or were interfering in any way with the accuracy of the test. These tests consisted in titrating two samples of water from a common source and of the same volume, one of which contained a quantity of feces. Although this test was repeated several times, no differences were detected in the oxygen content of the water, showing that the presence of feces did not interfere with the accuracy of the test. In a like manner, the possible iodine absorption power of the integument of the larvae was tested with negative results.

TABLE 2

Average oxygen consumption in cubic centimeters per gram weight

NUMBER TESTED	STAGE								AVERAGE INCREASE
	1	2	3	4	5	6	7	8	
32	.157	.233							.076 ± .015
19	.160		.254						.094 ± .012
33	.160			.282					.122 ± .011
36	.178				.286				.108 ± .011
16	.171					.264			.093 ± .019
27	.174						.298		.124 ± .011
10	.192							.344	.152 ± .013

Thirty-two stage 2 larvae were tested in all, which gave an average oxygen consumption of 0.233 ± 0.009 cc. oxygen per gram weight (table 2). The same thirty-two larvae consumed but 0.157 ± 0.012 cc. oxygen per gram weight during stage 1, which fact showed that an average increase of 0.076 ± 0.015 cc. oxygen per gram weight had been attained by the time stage 2 was reached, or an increase of 48 per cent. It may be mentioned here that the per cent increase computed for each succeeding stage of metamorphosis was based on the oxygen consumption of the individuals tested as compared to what the same specimens consumed during stage 1. Since each stage tested involved a different grouping of the larvae, it is clear that the average stage 1 oxygen consumption used for comparison would vary to a certain extent as the different stages were considered.

The following six stages showed in general a progressive rise in oxygen consumption over their stage 1 consumption. The nineteen stage 3 and the thirty-three stage 4 larvae showed a respective increase of 59 and 76 per cent. Stages 5 and 6 showed a somewhat lessened increase over the normal or stage 1 consumption as compared with stage 4, or 61 and 54 per cent, respectively. The last two stages (7 and 8) gave respective increases of 71 and 79 per cent over the normal or stage 1 consumption, stage 8, as was expected, owing to the advanced stage of metamorphosis, possessing the greatest increase per gram weight of any stage tested. The calculation of the probable errors showed that the differences between the means of the various stages and their stage 1 means exceeded the probable errors of their differences from five to twelve times. This then obviates the possibility that the rise in oxygen consumption might have been due to random sampling.

Table 2 gives the average amount of oxygen in cubic centimeters consumed per gram weight in one hour for the eight stages, while text figure A represents graphically the per cent increase in oxygen consumption of the same individuals. The unexpected lowering of the oxygen consumption during stages 5 and 6 and other related factors which probably influenced the oxygen consumption at various stages will be taken up later under the discussion of the results in general. It is sufficient to state here that, in general, a progressive rise in oxygen consumption was found to occur as the larvae attained each successive metamorphic stage.

EFFECT OF ETHERIZATION ON OXYGEN CONSUMPTION

The fact that stage 1 larvae were anaesthetized with ether prior to the testing period introduces the possibility that such treatment may have influenced their oxygen consumption and thereby introduced serious errors in computing the per cent increase or decrease as compared with later unanaesthetized stages. Should the oxygen consumption of normal larvae be considerably reduced by the ether, it is evident that the significance of the results previously described would be greatly minimized if not rendered worthless entirely. For these reasons it was very essential that the exact effect of ether on the respiratory rate be determined.

Sixteen normal tadpoles were selected from the same stock and of the same size and degree of development as those employed in the stage 1 oxygen tests. The oxygen consumption of these were then determined by individual tests. At the completion of the one-hour testing period the same individuals were anaesthetized in a 2 per cent aqueous solution of ether, using the same technique employed for all stage 1 larvae, and a second test run. In this way the results of the two tests could be compared and the changes in oxygen consumption computed. The average consumption of the sixteen unanaesthetized specimens tested was 0.172 cc. oxygen per gram weight per hour, while the average consumption of the same sixteen with etherization was 0.205 cc. oxygen per gram weight per hour. This gives an increase of approximately 19 per cent due to etherization.

In order to see whether the effects of etherization extended over a period of several hours or were only temporary, a second set of tests were carried out. This involved the testing of the same sixteen individuals three hours after the completion of the first etherization test. Immediately following this third test, the animals were etherized for the second time and tested again. The results of these two tests showed that the animals had only partially recovered from the effects of the first etherization and were consuming 0.196 cc. oxygen per gram weight per hour, or 14 per cent more than they were before the first anaesthesia was administered. The second etherization test showed that 0.218 cc. oxygen per gram weight per hour were being consumed on the average, or 11 per cent more than the second normal test and 27 per cent more than the first normal test. In other words, the second etherization raised the oxygen consumption 8 per cent higher than the first application of ether.

The relation of these results to the oxygen consumption of stage 1 larvae, as determined in the present experiments, is of considerable importance. It seems safe to conclude, for example, that had it not been advisable to employ etherization to reduce activity among stage 1 larvae when tested, the oxygen consumption of the latter would have proved to be at least 10 per cent lower than calculated. This fact would increase the differences that were shown to exist between larvae in different stages of metamorphosis and those of stage 1.

DISCUSSION

A consideration of the data presented in this paper leaves no doubt that a more or less steady and progressive increase in metabolic rate parallels the transformation stages of R. pipiens larvae. There are, however, a few points at which the rate is unduly accelerated or retarded as compared with the expected rate. It will be noted in this regard that 48 per cent of the total 79 per cent increase as recorded occurs between stages 1 and 2, at which time the larvae have undergone but slight external metamorphic changes. This initial abnormal increase is due largely to the marked decrease in body weight amounting to 32 per cent. Such a decrease in body weight must, therefore, be attributed to other causes than degenerative processes and can be explained in a large measure by the loss of water which is particularly marked during the early stages of metamorphosis (Yung, '85). The falling off of the metabolic rate during stages 5 and 6, on the other hand, seems to be due to the general health conditions of the larvae during these periods. It was consistently noted that, when the transforming larvae arrived at these stages, they became quite weak and inactive and in many cases died within a few days. Evidently, these stages represent the critical periods in di-iodotyrosine metamorphosis, since normally metamorphosing tadpoles do not generally exhibit like weaknesses during similar periods of their development.

The general weakness observed in the di-iodotyrosine larvae was usually accompanied by an edematous condition. This, according to Jordan and Speidel ('23), is associated with a deficiency of corpuscular elements resulting from the inadequate development of the blood-forming organs, notably the spleen. As previously noted, di-iodotyrosine metamorphosis does not permit of the full and normal occurrence of the various metamorphic changes either as regards rate of development or degree of transformation. The rather large variations in metabolic rate found to exist between individuals of the same age and metamorphic stage can be attributed largely to such abnormalities.

The results of etherization of stage 1 larvae are of interest in viewing the rise in metabolic rate. Etherization increased the oxygen consumption of this stage and therefore produced a much too high figure for comparison with the other stages in which anaesthetization was not necessary. Correction made for this error would augment the differences computed between stage 1 and the succeeding stages by approximately 19 per cent.

The comparison of the increase in metabolic rate with the decrease in total weight (text figure A) is of considerable interest in determining whether the oxygen consumption increases absolutely (i.e., per gram weight) or relatively (i.e., per individual) during each stage. In general, it may be said that at all stages tested, with the possible exception of stage 6, the increase is both absolute and relative, since the larvae consume on the average an increasing amount of oxygen regardless of the decrease in body weight. These results correspond, therefore, with Groebbel's ('22) findings on thyroid metamorphosing R. temporaria larvae, in which he found an absolute and relative increase in oxygen consumption. The fact that Abelin and Scheinfinkel ('23) found a decrease in carbon-dioxide production occurring in R. esculenta larvae, amounting to 50 to 70 per cent near the end of transformation, does not seriously conflict with the conclusions reached in the present paper, since the former authors were concerned only with the amount of carbon dioxide produced per animal. As far as the writer is aware, no weighings were made or attempt made to calculate the carbon-dioxide production per gram weight. It may well be, therefore, that the decrease in weight of the R. esculenta larvae tested was more than 50 to 70 per cent, which, if true, would show that although the larvae were producing 50 to 70 per cent less carbon dioxide at this time, they were actually producing more carbon dioxide per gram weight, and hence had increased their metabolic rate.

The significance of the increases in metabolic rate prevalent during the metamorphosis of anuran larvae is one that should not be overlooked. The fact that such an increase occurs at the onset of transformation would seem to be due, at least indirectly, to the influence of the thyroid hormone. In fact, Jarisch ('20) found that if thyroid feeding was accompanied by a diet which would decrease the protein metabolism, metamorphosis would be retarded. This fact would indicate, therefore, that the thyroid normally possesses a stimulating effect on metabolism. The exact relation of the heightened metabolism to the metamorphic changes occurring is still, however, a matter largely of opinion. It is just possible that certain organs or parts destined to undergo transformation or development can so react only when a certain rate of metabolism is reached. The fact that the various metamorphic changes do not occur at the same time, but always in a certain definite order and associated with a more or less definite metabolic rate, would seem to indicate that such is the case.

There are, however, certain strong objections to the acceptance of such a theory in that considerable variations in metabolic rate are a common occurrence among individuals of the same size and stage of metamorphosis. The writer[1] has also found that great specific differences exist between the metabolic rates of Amblystoma larvae which metamorphose at approximately the same time and that neotonous A. tigrinum larvae (axolotl) have as high a metabolic rate as the normal A. tigrinum larvae which undergo metamorphosis. Furthermore, the metabolic rate of most amphibians may be altered by temperature, anaesthesia, food, etc., without inducing metamorphosis.

The writer is inclined to regard the changes in metabolism that occur more in the light of secondary phenomena of metamorphosis not functioning as causative factors. According to this viewpoint, quite widely held by now, the thyroid initiates the transformation peculiar to metamorphosis either through the action of its own hormone or through the medium of other secretory substances brought into action, released, or formed, through its presence. Such indirectness of action has been conclusively shown by the writer ('26) to be the case in the formation of the fore-leg perforations, where the atrophying gills undoubtedly produce a substance responsible for the histolysis of the opercular integument of that area. Changes in metabolic rate would seem to be due, therefore, to the degenerative and constructive processes brought into play during metamorphosis, the increased metabolic rate simply indicating that the anabolic processes are in excess of the katabolic processes.

SUMMARY AND CONCLUSIONS

1. Over five hundred Winkler oxygen-consumption tests were made on larvae of R. pipiens in various stages of metamorphosis in an attempt to determine the changes in metabolic rate that occur.

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[1] The metabolic rates of Amblystoma larvae. Jour. Exp. Zoöl. (In press.)

2. A progressive increase in oxygen consumption per gram weight was found to occur, amounting to approximately 79 per cent in stage 8 as compared with the normal or stage 1 larvae.

3. R. pipiens larvae metamorphosed by di-iodotyrosine undergo a gradual reduction in weight amounting to 57 per cent in stage 8. Di-iodotyrosine, as a metamorphosing agent, was found to be very efficient, although inducing a type of metamorphosis somewhat different from the natural.

4. Etherization of normal larvae (stage 1) was found to increase oxygen consumption approximately 19 per cent, which fact augments the percentage increases of oxygen consumption computed for the various succeeding stages of metamorphosis.

5. The increase in oxygen consumption during metamorphosis of R. pipiens larvae is both absolute (i.e., per gram weight) and relative (i.e., per individual), showing that there is an actual rise in the metabolic rate.

6. The increased metabolic rate found to exist is regarded by the writer as a secondary phenomenon of metamorphosis and not to be viewed as a causative factor of that process.

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PLATE 1

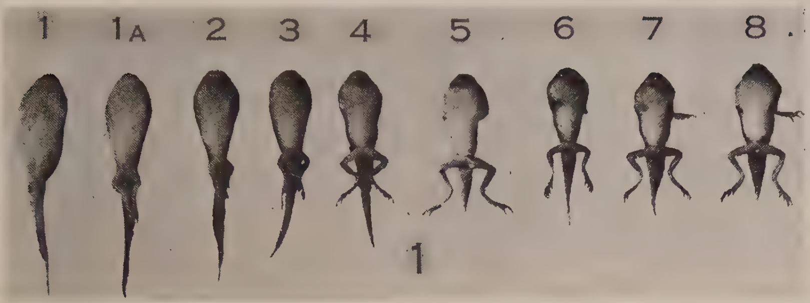
EXPLANATION OF FIGURES

1 Stages 1 to 8, inclusive, of di-iodotyrosine metamorphosis selected for the oxygen-consumption tests. Rana pipiens. × $\frac{2}{3}$.

2 Series of tadpoles undergoing natural metamorphosis. The stage numbers correspond to those of the di-iodotyrosine series pictured in figure 1, the comparison being made on the degree of fore-limb development. Rana pipiens. × $\frac{2}{3}$.

3 Dorsal view of first five stages of di-iodotyrosine series pictured in figure 1. Rana pipiens. × $\frac{2}{3}$.

4 Lateral view of first five stages of di-iodotyrosine series pictured in figure 1. Rana pipiens. × $\frac{2}{3}$.



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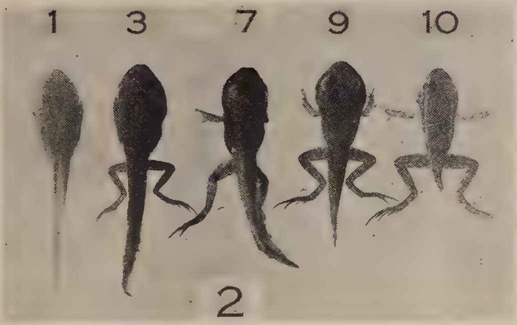

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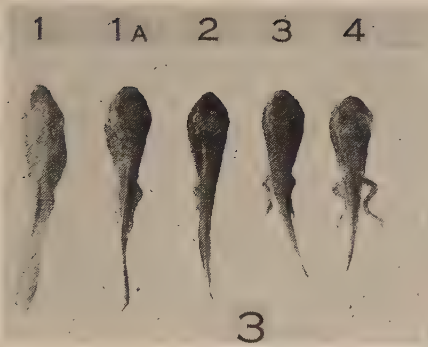

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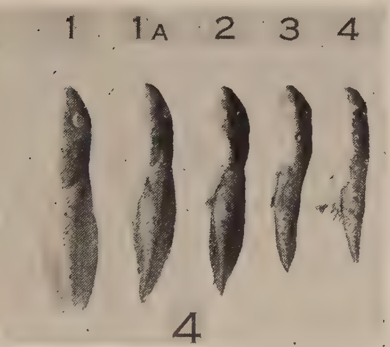

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TESTICULAR DEGENERATION IN ALBINO RATS FED A PURIFIED FOOD RATION

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ELEVEN FIGURES AND THREE CHARTS

CONTENTS

Introduction 159
Material and methods 163
  A. Dietary régime 163
  B. Technique 163
Histology of the testis 166
  A. Normal histology 166
  B. Pathological histology 167
Production and analysis of sterility on the basal ration 179
  A. Maintenance on the basal ration from weaning 179
  B. Maintenance on the basal ration after the attainment of sexual maturity 186
Attempted cure of the dietary sterility 190
  A. By return to stock-food diet 191
  B. By addition of lettuce to basal ration 191
Prevention of the dietary sterility 205
  A. By rearing on basal ration plus lettuce 205
Discussion 206
Summary 218
Bibliography 220

INTRODUCTION

From a survey of the literature dealing with the relation between sterility and nutritional factors we find that conditions of partial and complete sterility have been attributed to the following variety of factors: 1) Excessive meat diet (Paul, '06, and Hewer, '14, in rats). 2) Lipoid-free diet (Hatai, '15, in rats). 3) Partial and total inanition (Grandis, '89, in pigeons; Loisel, '01, and Poikarov, '13, in the dog; Siperstein, '21, in rats). 4) Lack of vitamines in deficiency diseases (Funk and Douglas, '14, in pigeons; McCarrison, '19, '20, in monkeys and pigeons; Allen, '19 a, in rats; Dutcher and Wilkins, '21, in cockerels; Meyerstein, '22, and Eckstein, '23, in rats). 5) Lack of sufficient protein or calcium (Reynolds and Macomber, '21, '21 a, in rats). 6) Milk diets or rations whose protein and vitamine content is supplied by whole-milk powder (Mattill and Conklin, '20; Mattill and Stone, '23; Palmer and Kennedy, '23; Anderegg, '24; Anderegg and Nelson, '25, and Sure, '24, in rats). 7) Purified diets containing adequate amounts of the growth vitamines (Osborne and Mendel, '19; Evans and Bishop, '22, '23 a; Evans, '25, in rats).

Successful reproduction has been obtained under the following conditions: 1) Diet of polished rice supplemented by green alfalfa (Dutcher and Wilkins, '21, in cockerels). 2) Synthetic diets with sufficient calorie value (Sugiura and Benedict, '23, in pigeons). 3) Whole-milk powder, in properly balanced 'purified' ration (Anderegg, '24; Anderegg and Nelson, '25). 4) Whole wheat and whole milk in proper proportions (Sherman and Campbell, '24, in rats). 5) 'Purified diets' supplemented with lettuce, wheat germ, alfalfa, meat, etc., to supply the vitamine E (Evans and Bishop, '22, '23 a; Mattill, Carman, and Clayton, '24; Miller and Yates, '24, and Evans, '25, in rats).

Previous to the work of McCarrison ('19, '20) on deficiency diseases and that of Reynolds and Macomber ('21, '21 a), the descriptions of atrophy and histological changes in the reproductive organs, resulting from defective diets, were rather meager, and very little account was taken of the many factors involved. Most observations on the male consist only of notes on the relative size of the testes, normality of spermatozoa, or breeding data. A more complete survey of the literature on this subject and a good description of the histological changes in the testis of the rat after acute and chronic inanition are given by Siperstein ('21).

Osborne and Mendel ('19) noticed that "although animals fed on artificial diets had grown vigorously to adult size they were, with very few exceptions, sterile." Except for the rather limited observations of Paul ('06), Funk and Douglas ('14), and Hewer ('14), this is the first indication in the literature that certain dietary régimes may be adequate for normal growth and development, but not for the normal reproductive processes. An examination of some of these rats, made by Allen ('19 a), showed "total degeneration of all the germ cells" in the testes. Since these rats had had a somewhat varied dietary régime, they afford no real conclusion as to the dietary factor or factors responsible, although Allen has attributed this to lack of the water-soluble vitamine B. The degenerative changes in the testes of rats described by Meyerstein ('22) and Eckstein ('23) also give little clue to the underlying cause, for the diets used by both are lacking in several factors. Cordes ('98) has described a similar degeneration of the testis after acute and wasting diseases. It is not unreasonable to assume that the testicular degeneration attributed to lack of specific substances in the diets may be due rather to a generally lowered vitality and subnormal condition of the body as a whole accompanying undernutrition or faulty metabolism.

Dutcher and Wilkins ('21) were the first to show that the reproductive failure may be improved or prevented by the addition of specific substances to an inadequate diet. They failed to get development of the testes of cockerels fed a diet of polished rice, but supplementing the rice with small amounts of green alfalfa prevented testicular atrophy. Likewise, alfalfa feeding increased the weight of atrophied testes, resulting from thirty-six days' rice feeding.

The greater part of the work on the relation of the diet to sterility has dealt chiefly with the female, and the evidences for sterility have been based principally on breeding tests. More recent histological studies (Evans and Bishop, '23, '23 a; Mattill, Carman, and Clayton, '24) on 'sterile' females fed on a synthetic ration have shown a normal histological condition of the ovaries (i.e., normal maturation and ovulation of the egg), the infertility being due to improper implantation or placental nourishment, which results in abortion or resorption of the embryos. From experiments with natural foodstuffs, Evans and Bishop were led to postulate the existence of a new factor, necessary for normal reproduction, at first termed vitamine X, but now classed as vitamine E (Evans, '25). They have found this abundant in lettuce, whole wheat, meat, alfalfa, etc., and have succeeded in obtaining this vitamine in a very concentrated form (Evans and Burr, '25).

Osborne and Mendel ('19) had noted the reproductive failure in many of their rats fed rations composed partially or totally of purified food substances, and at their suggestion Mr. J. T. Thompson, of the Osborn Zoölogical Laboratory, began a histological study of these rats in 1921. His results from the study of the females were similar to those described by Evans and Bishop ('22) and histological studies of the testis of the male revealed conditions similar to those described by Allen ('19 a). Unfortunately, Mr. Thompson was forced by severe illness to discontinue his work in May, 1922.

In September, 1922, the present writer took up the work, with the view of making a detailed histological study of the degeneration of the testis in male rats, fed an artificial diet adequate for growth, but not for reproduction, and of determining the possibilities of the prevention and cure of the disorder by supplementing the diet with other food substances. The results of this work are contained in the present paper.[1]

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[1] The writer wishes to express his thanks and indebtedness to Dr. R. G. Harrison, under whose direction the histological studies of this problem were carried out at the Osborn Zoölogical Laboratory; to Dr. T. B. Osborne and Dr. L. B. Mendel for their encouragement and cooperation in connection with the dietary work, the expenses of which were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C., and to Miss Helen C. Cannon for her assistance in the care and supervision of the experimental animals.

MATERIAL AND METHODS

A. Dietary régime

Of the numerous dietary régimes used by Osborne and Mendel in their experiments on animal nutrition, the 'standard' casein diet has proved very successful as far as the growth and vigor of the rats are concerned, although absolutely inadequate for reproduction. For this reason, this diet was adopted as the basal ration for the study of the infertility of rats reared under such a régime. The composition of this diet is as follows:

Basal ration[2]

	Per cent
Casein,	18
Corn-starch,	54
Lard,	15
Milk-fat,	9
Salt mixture,	4
0.2–0.4 gram of dried yeast daily	

This diet is identical with the basal ration (Standard Diet II) used by Evans and Bishop ('23 a) in their valuable contribution on the dietary sterility in female rats, and in more recent studies on the males (Evans, '25).

All the experimental animals were weighed biweekly and the growth curve of each charted. The food intake was also measured and charted along with the growth curve, with notes on the food spilled, if any, and the condition of the feces. The cages were changed and sterilized at each weighing.

B. Technique

Operative. Of the 130 male rats used in this study, ninety-seven were subjected to unilateral castration. The operative procedure was undertaken for four reasons: First, to make

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[2] Since diets of the same composition may show appreciable differences of purity, depending on the method of obtaining and purifying the various ingredients of the diet, especially the protein, the sources and method of preparation of the various ingredients of the basal ration as used in these experiments will be described.

Casein. Prepared from skim milk as follows: 1. Skim milk plus HCl. Usually 6 liters of HCl (made by diluting 250 cc. concentrated HCl with 10 liters of a more accurate study of the effect of the diet, after different periods of feeding, by means of a comparative histological study of the two testes of the same rat taken at different periods, thus eliminating to some extent the factor of individual variation of the rats in their reaction toward the dietary régime. Secondly, to study twice as many testes, at various stages of treatment, as would otherwise be possible with the same number of rats. Thirdly, to help in determining the rapidity of the process of degeneration, by comparing

water) to 40 quarts of milk in a large kettle with a capacity of about 80 quarts (165 liters), to precipitate the casein.

2. Drain over cheese-cloth and press in hydraulic press (2000-lb. pressure).

3. Pass through meat chopper to break up lumps. Then pass with water through the finest disc of chopper.

4. Dilute with two to three times the volume of water (to about 45 qts.).

5. Dissolve in about 250 cc. of NaOH (250 cc. concentrated NaOH to 10 liters of H_2O). Dilute with water up to limit of vessel.

6. Precipitate with acetic acid (made up with 200 cc. concentrated acetic acid to 10 liters of H_2O).

7. Drain, press, and grind with water as before.

8. Dissolve in NaOH as before.

9. Precipitate with HCl as before.

10. Wash with water, usually by spreading on rack, and washing repeatedly with stream from hose.

11. Press, grind, and dry.

12. Grind to fine powder.

Starch. Commercial cornstarch as put up in 1-lb. lots by the Corn Products Co., New York.

Lard. Commercial leaf lard, as produced by Sperry & Barnes Co., New Haven, Connecticut.

Milk-fat. Commercial tub butter. The butter was melted, the water separated out, and the fat filtered in a warm charmber.

Salts. The mixture used was the lactose-salt mixture, based on the composition of salts in milk, as described by Osborne and Mendel ('18). The composition of the mixture is as follows:

	Grams		*Grams*
$CaCO_3$,	13.48	Citric acid, H_2O,	11.11
$MgCO_3$,	2.42	Ferric citrate, $1\frac{1}{2}$ H_2O,	0.634
Na_2CO_3	3.42	KI,	0.002
K_2CO_3,	14.13	$MnSO_4$,	0.0079
H_3PO_4,	10.32	NaF,	0.0248
HCl,	5.34	$K_2Al_2(SO_4)_2$,	0.00245
H_2SO_4,	0.92	Lactose,	246.0

The salts used are all of the highest degree of purity obtainable.

the histological condition of the testis removed at operation and at autopsy, and noting the changes undergone in the intervening period. Fourthly, to secure accurate data on recovery, by determining the condition of one of the testes before the animal was transferred to the recovery diet.

All the operations were performed under ether anaesthesia with aseptic precautions. All the animals recovered readily and not a single case of subsequent infection was observed. This absence of infection is a good indication of the healthy, vigorous condition of the animals and the sanitary condition under which they were kept.

In a few cases (young rats in which neither testis had descended) an incision was made through the abdominal wall and the testis removed. In practically all other cases the left testis was removed by means of an incision on the ventral wall of the left half of the scrotum. In a few cases where the right testis was descended, but the left still abdominal, the operation was performed on the right side instead. Except in the earlier operations, before the value of such data was fully realized, careful notes were taken on the macroscopical appearance, dimensions, and weight of the testis before fixation.

Histological. Before immersion in the fixative, the testis was snipped into smaller pieces to insure good fixation. In practically all cases at least two different fixatives were used, the anterior half of the testis dropped into one fixative and the posterior half and epididymis into the other. Usually one half was fixed in Bouin or Allen's B-15 (Allen, '19) and the other half in Zenker or Helly's formol-Zenker. In the case of Bouin and B-15, fixation was considerably improved by warming the fixative at 40°C. for the first hour of fixation. The picric acid was removed readily with the aid of lithium carbonate in 70 per cent alcohol. Regaud and Flemming fixatives were also used in a number of cases.

After dehydration, the tissues were cleared in xylol or cedar oil (the latter proving particularly satisfactory) and embedded in paraffin with a melting-point of 56° to 58°C.

Sections were cut 4 to 7 μ in thickness and mounted for staining. Sections were made of portions of both the anterior and posterior halves of each testis, which had previously been subjected to different fixation. This was done in order to detect any slight differences which might be due to fixation alone, as well as to determine whether the histological changes found were uniform in both portions of the testis.

Practically all preparations were stained in Heidenhain's iron hematoxylin, both with and without counterstaining with orange G. A number of slides were also stained with Delafield's hematoxylin and eosin, Mallory's triple stain and eosin methylene blue. Heidenhain's iron hematoxylin gave most satisfactory results for both general and detailed study.

HISTOLOGY OF THE TESTIS

A. Normal histology

The descent of the testis into the scrotum occurs in the rat at about the fortieth day of life, which is also about the time of ripening of the first spermatozoa. In the adult rat each tubule is constantly active in the production of spermatozoa up to old age, as is characteristic of all mammals. The detail of the process of spermatogenesis in the rat has been carefully studied by various workers, particularly Brown ('85), Regaud ('01), v. Ebner ('02), Duesberg ('08), and Allen ('18). In general, the descriptions agree closely with those given for other mammals by many other investigators. Since it is not the purpose of this paper to discuss to much extent the more detailed cytological changes, a general description of spermatogenesis in the rat will suffice.

The seminiferous tubules of the testis, which are surrounded by an endothelial wall (the membrana propria), are lined by a stratified epithelium, usually termed the 'germinal epithelium.' This epithelium normally bears four types of germ cells: *a*) spermatogonia, forming the basal layer, which divide and undergo a period of growth to produce the larger, *b*) spermatocytes, which undergo mitotic division and chromosome reduction to form the, *c*) spermatids, which in turn elongate and transform into the, *d*) mature spermatozoa. Besides these, a fifth type of cell, the so-called 'Sertoli' or 'nurse' cell, is found resting on the membrana propria. These are really a syncytium of Sertoli nuclei in a cytoplasmic mass, in which the real germ cells are embedded and which is intimately connected with the attachment and nourishment of the developing spermatozoa.

In brief, spermatogenesis consists of a wave-like activity passing along the seminiferous tubule in such a manner that in one part of the tubule early stages of spermatocytes and spermatids may occur (fig. 1 and fig. 7), while in another part dividing spermatogonia and nearly mature spermatozoa may appear. In other parts various combinations may be found. This cycle in the rat repeats itself about every 32 mm. of tubule length (v. Ebner, '02).

Figure 1 shows a number of tubules from a normal testis in the different phases of the spermatogenic cycle. Three of the tubules show spermatozoa in normal numbers, with the tails extending free into the lumen. Three other tubules (left) contain immature spermatozoa which have not yet developed tails, and one tubule (upper right) shows developing spermatids in large numbers. One of the above tubules containing immature spermatozoa (lower left) also shows numerous mitotic divisions of the spermatocytes.

B. Pathological histology

Rats fed for sufficient time on the standard casein diet (i.e., the basal ration), either after weaning or after attainment of sexual maturity, have shown a progressive degeneration of the germinal epithelium of the testes, regardless of their excellent growth and healthy condition. The time required to induce the testicular degeneration and the length of the process, together with the possibilities of preventing and curing the disorder, will be discussed in the later part of this paper. The present section deals with the histological changes involved.

Of the 220 testes from experimental and control rats used in this investigation, 140 have shown various stages of degeneration of the germinal epithelium of the seminiferous tubules. From a study of these histopathological pictures it has been possible to follow all the steps in the progressive degeneration from the normal to a condition involving complete loss of the germ cells. For convenience, the process of degeneration has been divided into five distinct stages.

It is necessary to emphasize that, although sometimes the seminiferous tubules of a single testis may show practically the same stage of degeneration, this is not usually the case. As a rule, some tubules seem more resistant to degeneration than do others, resulting in a rather irregular retrogression of the germinal epithelium in different tubules, so that the testis may show several stages of degeneration at the same time. However, one stage usually is the predominant one, and in the experimental part of this work the testes of different animals have been classified according to their predominant stage of degeneration.

Stage 1 (figs. 2 and 7). This stage is characterized by the degenerative conditions of the mature spermatozoa, but a normal appearance of the rest of the germinal epithelium.

Figure 2 shows five adjoining tubules, two of which (left) are producing normal spermatozoa in contrast to the other three (right) exhibiting distinct sperm degeneration. The spermatozoa undergo chromolysis, eventually fusing together in very irregular masses. These conditions can be observed readily under low power of the microscope.

Figure 7 shows an enlarged view of a portion of the germinal epithelium (*X*) of one of the tubules of figure 2. It will be noted that the compact epithelium contains perfectly normal spermatogonia (*SG*), spermatocytes (*SP*), and spermatids. The spermatid nuclei (*SN*) are in early stages of elongation preparatory to their transformation into spermatozoa. Two large Sertoli cell nuclei (*SC*) may be seen just within the membrana limitans, and extending from these toward the lumen are delicate cytoplasmic strands—the cytoplasm of the Sertoli syncytium, which fills the spaces throughout the epithelium not occupied by the germ cells proper—to which the young spermatids normally attach themselves for support and nourishment during their transformation into mature spermatozoa. As these spermatozoa mature and pass toward the lumen, their heads are still embedded in the Sertoli syncytium, which is usually drawn out into the fibrous processes as mentioned above. These processes represent the previous cytoplasmic attachment of the crop of spermatozoa (*DS*) which are showing the degenerative changes at this stage.

There is no doubt that the mature spermatozoa are the first of the germ cells to show signs of deterioration, as might be expected, because of their sensitiveness and highly specialized structure. Then, too, if the degeneration be due to a nutritive lack in the blood stream, those cells more remote from the vascular sources would naturally be the first to exhibit this lack. The earliest indication of an abnormal condition of the spermatozoa is a knot-like bulging just behind the head region, followed by a swelling of the head piece itself. This is clearly seen in stained smears from the epididymis of testes at this stage of degeneration. As chromolysis continues, the spermatozoa gradually lose their individuality. Those associated in groups soon show a coalescing, resulting in the formation of the large irregular blotches or masses of chromatin material (fig. 7, *CM*) which are embedded for a time in the germinal epithelium. Many spermatozoa also pass into the lumina of the tubules and degenerate there. The tails of the spermatozoa later show degenerative changes similar to those of the sperm head and stain intensely with hematoxylin.

The degenerate spermatozoa and chromatin masses (fig. 7, *DS* and *CM*) are eventually cast off into the lumina and carried to the epididymis. At the end of this stage the tubules are practically free of spermatozoa, except for a few degenerate remains which are still embedded in the cytoplasm of the Sertoli syncytium. These often persist through several of the later stages and represent immature spermatozoa which were embedded deeply in the cytoplasm when the more mature spermatozoa were cast off.

From the above description it would appear that the normal process of spermatogenesis up to the formation of the spermatids has not yet been affected. It is, of course, impossible to tell how many crops of spermatozoa may undergo degeneration, but it seems doubtful that more spermatozoa are produced after the disappearance of the first degenerate group. That is, after the removal of the degenerate spermatozoa seen in figure 7, the spermatids which appear normal would have lost their ability to undergo transformation into spermatozoa. There is, then, a lack of normal vigor in the germ cells which prevents them from continuing their usual spermatogenic activities. This view is confirmed by a study of the next stage.

Stage 2 (figs. 3 and 8). This stage, which is of relatively short duration, is characterized by almost complete absence of spermatozoa and by a peculiar bead-like appearance of the spermatid nuclei, which is quite evident even at low magnifications.

Figure 3 shows the general appearance of tubules at this stage. Two tubules nearest the center are beginning to exhibit the multinucleate condition found in the following stage. The other tubules, however, show a uniform type of degeneration belonging to this stage and differ considerably from those of the previous stage. In addition to the absence of spermatozoa and the large number of bead-like spermatid nuclei, the germinal epithelium itself is rather swollen and edematous, reducing the size of the lumen completely in one and to a considerable extent in the others. Numerous vacuolated spaces have also appeared within the epithelial layer.

Figure 8 gives an enlarged view of a small portion of the germinal epithelium (*X*) of one of the tubules shown in figure 3. This represents a slightly late phase of this stage, but was selected for the drawing, since it is very useful in explaining the peculiar condition found in the following stage.

Examination of the epithelium of a tubule at this stage, such as shown in figure 8, reveals normal spermatogonia (*SG*), spermatocytes (*SP*), and Sertoli cell nuclei (*SC*). The epithelium itself, however, presents a greatly swollen appearance with the cells rather loosely scattered and surrounded by vacuolated spaces or areas of less dense cytoplasm. This has resulted from plasmolysis of the cytoplasm, especially that forming the Sertoli syncytium, and affords marked contrast with the normal condition of the epithelial layer seen in figure 7.

But equally striking is the beaded or vesicular appearance of the spermatids[3] at this stage. Normally, the nuclear material is evenly distributed throughout the nucleus and has a rather faint staining reaction. The nuclei of the degenerating spermatids at this stage (fig. 8, *SN*) appear bead-like or ring-like on superficial examination, but careful focusing reveals a hollow spherical nucleus with many small and irregular deeply staining particles of chromatin lying close to the nuclear wall. As degeneration proceeds, these particles lose their more regular distribution and become condensed at one side of the nucleus in a deeply staining crescent-shaped area.

At the same time, plasmolysis of the surrounding cytoplasm takes place, the cell walls of the spermatids disappear, and the cytoplasm of adjoining spermatids becomes more or less continuous. Some of these cytoplasmic connections may be seen at *B*, figures 8 and 9. Later, the cytoplasm of many of these degenerating spermatids becomes more and more continuous until large irregular masses of cytoplasm are formed containing from two to many spermatid nuclei. These cytoplasmic masses are the forerunners of the giant cells so characteristic of the following stage.

Figure 8 (*GC*) shows an excellent example of such a condition. This large homogeneous mass of cytoplasm still exhibits a number of connections with the surrounding cytoplasm, but contains seven spermatids whose cytoplasm has fused completely. The nuclei (*SN*) of the spermatids show the crescentic clumping of the chromatin, and the so-called chromatoid bodies (*CB*) of five of the seven spermatids are clearly seen. Three of these bodies are already undergoing chromolysis, as indicated by their swollen condition. In some cases it appears as if large areas of the germinal epithelium, containing many spermatids in process of degeneration, simply separated in masses from the surrounding layers and later rounded off to form the anlage of the giant cells of the next stage.

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[3] Since it is difficult, if not impossible, to distinguish between secondary spermatocytes and spermatids; many of the cells which I have considered spermatids may be spermatocytes of the second order.

Not all the spermatids have this fate, for a great many pass off into the lumina of the tubules before the breakdown of their cellular wall and consequent fusion of their cytoplasm. Such cells accumulate in large numbers in the lumina and undergo considerable liquefaction there before reaching the ducts of the epididymis, where they may be found packed together in large dense masses and showing all degrees of pycnotic and autolytic changes.

The conditions seen in the above stage support the view previously stated—that for a considerable period, at least, the germ cells are capable of undergoing their normal maturation and development up to the spermatid stage but, lacking sufficient vigor to complete the last stage of the spermatogenic process, the degenerative changes set in. The spermatocytes certainly do undergo repeated division for a considerable time after the tubule has reached the condition of stage 1, as indicated by the large number of spermatids in the germinal epithelium and by the occasional mitotic figures found in the basal layers of tubules in the more advanced stages of degeneration.

Stage 3 (figs. 4 and 9). This stage has been termed the 'giant-cell' stage because of the predominance of these large multinucleate masses, which are really syncytia of from two to forty or more spermatid nuclei. Figure 4 shows a group of tubules from a single testis, four of which show the giant-cell condition. These cells stand out very distinctly from the rest of the epithelial layer and show somewhat different degrees of staining capacity, depending on the amount of pycnosis which they have undergone. Two of these tubules (lower left and upper right) show groups of degenerating spermatids and occasional abnormal spermatocytes which have been liberated from the epithelium and have accumulated in the lumina. These cells are soon passed on to the epididymis unless they become entangled in the fibrous cytoplasm and degenerate spermatozoa remains often present. One tubule (lower middle) shows a considerably more advanced stage, with complete removal of the giant cells and the degenerate sperm remains, and shows many primary spermatocytes undergoing karyorrhexis. This condition will be described more fully in connection with the next stage.

There is no marked reduction in the size of the tubules, but the testes at this stage of degeneration begin to show an appreciable loss in weight, due to the great proliferation of spermatozoa and spermatids during the previous stages. The testis from which the drawings of this stage were made weighed 0.80 gram, whereas the normal weight, based on the body weight of the rat, should have been 1.01 grams, according to Donaldson's figures for the normal. Other testes in a similar state of degeneration show approximately the same decrease in weight. The testes at this stage usually feel somewhat softer than those in a normal condition.

Figure 9, an enlarged view of the region *X* of figure 4, gives a good conception of the germinal epithelium at this stage. The spermatocytes (*SP*) and Sertoli-cell nuclei (*SC*) still appear normal. The latter appear smaller than those shown in previous stages, but this is due to the fact that they were cut through regions of smaller diameter, as shown by a study of the serial sections of this region. The epithelium is swollen to practically the same height as in the previous stage, but the vacuolated spaces (*V*) are more pronounced. The rather irregular spaces toward the lumen are caused by the separation and plasmolysis of the cytoplasm in these regions; but the more rounded vacuoles (*V*) in the basal layers are due to the complete dissolution of some of the germ cells which have undergone degeneration much earlier than their adjoining mates. No spermatogonia are present in this particular area, but they are present, in a normal condition, in other parts of the same tubule. A few bundles of degenerate spermatozoon tails (*DS*) may be seen in the outer portion of the epithelium, representing spermatozoa which were immature and deeply embedded in the Sertoli syncytium at the time of general degeneration of the other spermatozoa.

The giant cells so characteristic of this stage may now be described. The early differentiation of these was mentioned previously (p. 171), and one of these, in an early period of formation, was shown (fig. 8, *GC*). The above-mentioned cytoplasmic masses, containing variable numbers of spermatid nuclei, soon lose their cytoplasmic connections, round off, and form spherical masses which often show a rather ovoid shape as a result of pressure from the surrounding cells. Four large giant cells and a number of smaller ones are seen in figure 9, *GC*. Many tubules show all variations in the size of these cells, from those containing only two, three, or four nuclei to those enclosing as many as forty such nuclei. Two spermatids, which are much later in fusing than the others, are seen near the lumen. Their cytoplasmic connection (*B*) and chromatoid bodies (*CB*) are clearly shown. Above and to the left of these are two small giant cells, one showing two and the other three spermatid nuclei. These have already undergone considerable pycnosis, as indicated by the deeper staining reaction of their more condensed cytoplasmic and nuclear material when compared with that of the two cells in process of fusion or with that of the surrounding cytoplasm. The larger giant cells never arise through the fusion of the smaller ones, but are formed in the same manner as the latter, the only difference being that much larger numbers of spermatids are enclosed within the cytoplasmic masses, probably as a result of a more uniform plasmolysis of the cells at that particular point. The fate of these giant cells is the same.

At first the spermatid nuclei of the cells are irregularly distributed throughout the cytoplasm and the entire cell stains with the same intensity as the surrounding epithelium. As degeneration proceeds, these nuclei become arranged in a more definite order around the periphery of the cell, while the chromatoid bodies of the original spermatids pass toward the center of the cytoplasmic mass, where they soon undergo chromolysis and eventually liquefy. At the same time, both the cytoplasm and nuclei are becoming more and more pycnotic and show a much deeper staining reaction. The two large giant cells in the lower part of figure 9 show the marked contrast between the above conditions. The cell at the left is still slightly irregular in shape and has a light staining capacity, while the smaller and more spherical one at the right shows marked pycnotic changes. The nuclei (*SN*) in both cases are smaller and more condensed than those seen in the previous stage. The ultimate fate of these peculiar giant cells will be considered in connection with the next stage.

Stage 4 (figs. 5 and 10). This stage shows a marked contrast with the condition described above, as is shown by a comparison of figures 4 and 5. The epithelium of the tubules is now greatly reduced, spermatids are absent or practically so, and giant cells are rare or in advanced stages of dissolution. Figure 10 is an enlarged section of a tubule at this stage selected particularly to show the condition and appearance of the few giant cells still present. Large numbers of the giant cells of the previous stage, after undergoing severe pycnosis while embedded in the germinal epithelium, are set free in the lumina of the tubules. Here some of these undergo complete liquefaction, while others are carried to the epididymis to meet the same fate.

A few giant cells may remain more or less embedded in the epithelial layer and liquefy in situ. Two such cells (*GC*) are shown in figure 10. The larger one shows the enormous size which these giant cells often attain, although others considerably larger than this one are often found. The cytoplasm of these cells shows granular liquefaction and lipoid degeneration, the former condition being much more marked in the smaller cell. The larger spherical vacuoles in the cytoplasm represent the former position of spermatid nuclei which have undergone complete dissolution, while the smaller ones probably represent lipoid granules formed in the degeneration of the cell, but dissolved in the process of fixation. Other preparations showing similar conditions of the giant cells, but fixed with Flemming's fluid, indicate the presence of many small spherical lipoid granules in the degenerating cytoplasm, which stain intensely black with the osmic acid.

As a rule, the nuclei of the giant cells are the first to show signs of liquefaction, and different cells exhibit all stages of their gradual dissolution and loss of staining capacity, while the cytoplasm retains its compact pycnotic condition for a considerably longer time. These cells shown in figure 10 contain a number of nuclei which have not yet disappeared, although the surrounding cytoplasm of the cell, especially in the smaller one, shows rather advanced granular liquefaction. Just why some of these nuclei are more resistant to dissolution than are others has not been determined.

It is not until this stage is reached that the spermatocytes and spermatogonia begin to exhibit distinct degenerative changes. Very rarely, a few spermatocytes in the basal layers may be found in mitotic division, but these mitotic figures do not appear normal. They appear to reach the metaphase or early anaphase, but, lacking sufficient vigor to complete the division, undergo degeneration. In most instances, however, the spermatocytes exhibit karyorrhexis, i.e., rupture of the nucleus and subsequent deposition of irregular deeply staining masses of chromatin on the nuclear wall, as shown in one tubule (lower middle) of figure 4, and in another tubule (lower left) of figure 5.

A few of these degenerating spermatocytes often fuse to form giant cells of two, three, or four nuclei, in the same manner as described in the case of the spermatids. But the majority of these cells are liberated singly from the epithelium and pass to the epididymis where they complete their dissolution.

The spermatogonia rarely, if ever, form multinuclear cells, but, after partial degeneration in the epithelial layer, they are sloughed into the lumen and meet the same fate as the spermatocytes. A few of these in early stages of degeneration, as indicated by their swollen and vacuolated condition, are shown in figure 10 (*SG*).

The Sertoli-cell nuclei are the only elements of the germinal epithelium which have not shown degenerative changes. Those in figure 10 (*SC*) appear rather small, but this is due to the plane of sectioning having passed through only a part of the nuclei. After careful examination of a large number of testes at this stage, no abnormal conditions of these nuclei could be found.

Stage 5 (figs. 6A, 6B, and 11). This final stage of degeneration is essentially the same as that found in cryptorchid testes. All the germ cells of the germinal epithelium of the tubules have disappeared, and all that remains is a syncytium of Sertoli-cell nuclei in a greatly reduced layer of fibrous cytoplasm.

The general appearance of the tubules at this stage is seen in figures 6A and 6B. In figure 6A the tubules have not been greatly reduced in size in spite of the extreme loss of the germinal epithelium. It is remarkable that the tubules can retain to such an extent their normal shape and size under these conditions, for often an entire testis shows every tubule in this state. But in figure 6B the tubules show marked shrinkage to approximately half the normal size. Compare with the size of the tubules in figure 1, which is drawn under the same magnification. Both these conditions are sometimes found in the same testis, but usually in different parts of the organ. More often all the tubules of a single testis exhibit either one condition or the other.

The interstitial tissue becomes more prominent at this stage, due not to any hypertrophy of the tissue itself nor to any hyperplasia of the interstitial cells, as far as can be determined, but to the great decrease in the size of the tubules. That is, the apparent increase is not actual, but only relative to the size of the tubules. There is, however, a tendency for the interstitial tissue to accumulate in larger masses or clumps, as shown in figure 6B, possibly as a result of pressure from the intertubular fluid which has accumulated.

The true nature of the Sertoli syncytium is clearly seen in figure 11. This syncytium is very difficult to discern in normal tubules on account of the closely packed germ cells, although the Sertoli nuclei, which usually lie near the basement membrane, are easily distinguished by their large size, smooth but slightly flattened outlines, diffused chromatin material, and prominent nucleolus (plasmosome) with its closely associated but smaller 'paranucleolus' (karyosome). These nuclei have been mentioned in describing the previous stages and their normal condition noted. Even at this advanced stage of degeneration, at which all the germ cells have disappeared, the Sertoli nuclei still show a normal condition. It is true that many of these have been sloughed with the germ cells in the process of degeneration, and they are commonly found in the lumen associated with such cells, but this is not any indication of their degeneration. Seven of these large nuclei are shown in figure 11 (*SC*). These do not present the usual smooth outlines, for their membranes have become somewhat wrinkled and grooved, but their cytoplasm and nuclear material show no indications of degeneration. The syncytial cytoplasm, however, is more fibrous than normal.

We have seen, from the foregoing description, that male rats fed a diet producing excellent growth and a healthy vigorous bodily condition of the animals, may show a very marked process of testicular degeneration similar to that found in x-rayed testes, and to that produced by various pathological and experimental conditions.

The process, which has been divided into five arbitrary stages, consists of a gradual degeneration and removal of all the germ cells, until all that remains is the Sertoli syncytium. The germ cells degenerate in exactly the reverse order of their origin. That is, the spermatozoa are the first to disappear, next the spermatids, followed later by the spermatocytes and spermatogonia.

PRODUCTION AND ANALYSIS OF STERILITY ON THE BASAL RATION

A. Maintenance on the basal ration from weaning

The purpose of this series of experiments was to determine, by means of a histological study of the testes of a large number of rats fed the basal ration for different periods of time up to 150 days after weaning, 1) the degree of testicular degeneration produced after these various periods of dietary treatment; 2) the approximate time required to produce the disorder, and, 3) the time necessary to carry the process of degeneration to completion, i.e., the time interval between an early condition of stage 1 and the final condition of stage 5.

The more important data obtained from this series are summarized in table 1. A few words of explanation are necessary. The testis number in all cases bears the same number as the rat from which it was taken, followed by a or b, indicating that the testis under consideration is the one removed by operation (a) or the one taken at autopsy (b). A few, followed by ab, indicate that both testes were taken at autopsy without previous operation.

To show the splendid growth attained by these rats on the basal ration, the body weight of the rat at the time of removal of the testis is given as 'percentage normal by weight,' based on the figures of Donaldson ('15) for normal body weight on age. The normal testis weight is also computed from Donaldson's figures, and is based on the actual body weight of the rat. The actual weight of the testis is given, as well as the 'percentage normal by weight' of the organ, as calculated from the normal and the actual weights (in the earlier part of the work the testis weight was not ascertained).

The normal growth curve of the albino rat as given by Donaldson, as well as his figures for body weight on age and for testis weight on body weight, are slightly lower than similar figures for the normal rats of the Osborne and Mendel colony from which the rats used in this work were taken. However, since Donaldson's figures are so generally used, it was decided to base these computations on his figures. It will be necessary to bear in mind that the figures given in the tables for 'percentage normal by weight' of the rats and the testes are slightly higher than if the normal figures for our colony were used.

Chart 1 shows Donaldson's curve for the normal; the normal growth curve for our colony and the average growth curve of sixty-four experimental rats of this series fed the basal ration after weaning. It will be noted that the curve of the latter group is well above that of both the normal curves, showing that the testicular degeneration in such rats certainly cannot be attributed to lack of the necessary growth factors in the basal ration, and consequent undernourishment of the animals.

In table 1, as well as in the tables of the following series, the histological stage of degeneration is given for each testis studied. Because of the differential rate of degeneration of the tubules of the same testis, as has been mentioned before (p. 168), all of these tubules rarely show the same stage at the same time. Consequently, for convenience in tabulating and to avoid any unnecessary descriptions of individual cases, the degenerate testes have been classified and listed as being at that particular stage of degeneration which is found in the majority of the tubules, although other tubules may show greater or lesser degree of retrogression. Since the purpose of this and other series is mainly to determine the relative degree of degeneration obtained at the different periods of maintenance on the basal ration, such a method of classification is sufficient.

In this particular series, sixty-two rats, after the usual weaning period of twenty to twenty-five days, were fed the basal ration for various periods of time from twelve to one hundred and fifty days. Four of these rats were killed without operation and both testes taken for histological study, to see whether both testes of the same rat show the same condition at the same time, which they did. The remaining fifty-eight rats, after different periods of maintenance, had one of the testes (usually the left) removed by a unilateral castration. Fourteen of these were continued under the same régime for additional periods before autopsy and removal of the other testis. The other forty-four operated animals were put under a different dietary régime and will be referred to later in connection with the series dealing with the attempted cure of the testicular degeneration (p. 190). Thus, the testes from the four unoperated animals, the fifty-eight removed at operation, and the fourteen taken at autopsy give a total of seventy-six testes from rats fed the basal ration for different periods.

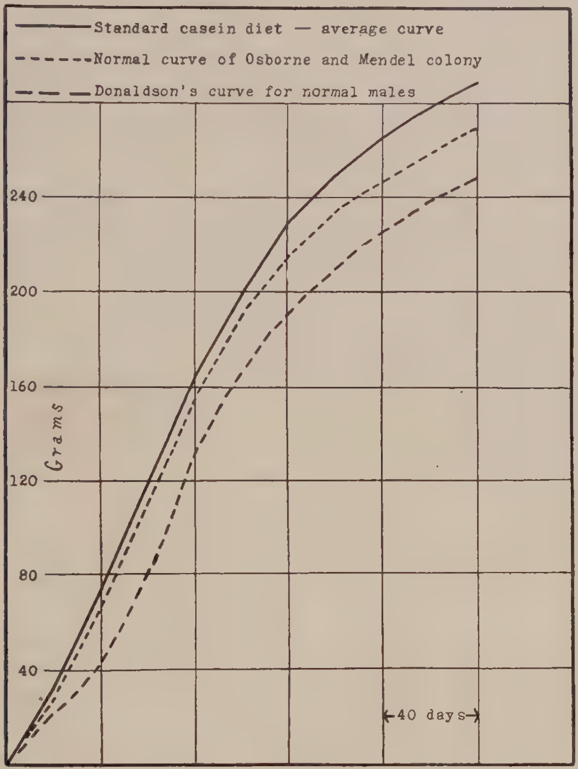

Chart 1 Showing the average rate of growth of experimental rats (fed the basal ration) as compared with the rate of growth of normal rats of the Osborne and Mendel colony and with Donaldson's curve for the normal male rat.

Table 1 gives complete data on the period of dietary treatment and relative weights of the rats used, together with

TABLE 1

To show body weight, testis weight, and histological condition of the testis of rats fed the basal ration for periods of 12 to 40, 50 to 55, 60 to 65, 70 to 75, 80 to 90, and 100 to 150 days after weaning

NO. OF TESTIS	PERIOD ON THE BASAL RATION	RAT PER CENT NORMAL BY WEIGHT	TESTIS WEIGHT			TESTIS—STAGE OF DEGENERATION
			Normal weight for actual body weight	Actual weight	Per cent normal by weight	
	Days		*Grams*	*Grams*		
9164ab	12	162	.....	.....	...	Immature
9165ab	16	157	.....	.....	...	Immature
8678a	33	141	.....	.....	...	Immature
9469a	40	133	.....	.....	...	Normal
Average		148				
B 526a	55	110	.893	.875	98	Normal?
B 540a	55	146	1.025	1.175	115	Normal?
B 812a	50	132	1.050	1.235	117	Normal
B 925a	50	125	.850	.900	106	Normal
9209a	50	100	.....	.....	...	Normal
9166a	50	101	.....	.....	...	1
9208a	50	129	.....	.....	...	1
9210a	50	123	.....	.....	...	1
9211a	50	125	.....	.....	...	1
8679a	50	110	.....	.....	...	1
9470a	50	92	.....	.....	...	1
B 859a	50	123	1.003	.880	88	1
B 872a	50	134	.920	1.260	137	1
B 924a	50	175	1.060	1.270	120	1
B 897a	50	154	1.010	.800	79	3
9172a	50	133	.....	.....	...	3
Average		126	Average		107	
B 639a	65	125	1.02	1.175	115	Normal
B 642a	60	138	1.12	1.25	112	Normal
B 644a	60	110	.93	1.04	112	Normal
B 648a	60	116	.96	1.06	110	Normal
B 607a	60	129	.93	1.24	125	Normal?
B 609a	60	123	.96	1.26	131	Normal?
8678 b	60	109	.92	.....	...	1
B 577a	65	134	1.07	1.18	110	1
B 579a	65	130	1.05	1.10	105	1
B 649a	60	96	.85	.77	90	1
B 723a	60	168	1.19	1.31	110	1
B1386a	60	128	1.03	.82	79	1
B1391a	60	105	.90	.73	80	1
B 614a	60	104	.92	.685	74	4
B 679a	60	115	.99	1.15	116	1
Average		122	Average		105	

TABLE 1—*Continued*

NO. OF TESTIS	PERIOD ON THE BASAL RATION	RAT PER CENT NORMAL BY WEIGHT	TESTIS WEIGHT			TESTIS—STAGE OF DEGENERATION
			Normal weight for actual body weight	Actual weight	Per cent normal by weight	
	Days		*Grams*	*Grams*		
B 245a	70	105	1.00	1.04	103	Normal
B 246a	70	102	.98	1.02	104	Normal
B 247a	70	106	....	....	...	Normal
B 53a	75	137	1.16	.93	80	Normal?
B 30a	75	126	1.12	.89	79	3
B 625a	70	134	1.09	.75	69	4
B 694a	75	124	1.11	.65	59	4
B 696a	75	136	1.16	.70	60	4
9469 b	75	113	....	....	...	4
9471a	75	130	....	....	...	4
9472a	75	126	....	....	...	4
B1492ab	70	110	.99	.74	64	Not studied
B1439ab	70	93	.91	.53	58	Not studied
Average 118			Average 75			
B 10a	90	114	1.14	1.10	96	Normal
B 255a	86	121	1.14	1.35	118	1
B 256a	86	117	1.12	1.21	108	1
B 257a	86	79	.87	.85	102	1
9987a	87	93	1.01	.86	85	1
B 260a	86	150	1.27	.91	72	3
8682a	80	102	....	....	...	4
8697a	80	138	....	....	...	4
B 252a	86	111	1.11	.65	59	4
B 253a	86	100	1.03	.52	50	4
B 251a	86	120	1.15	.65	43	4
B 617a	80	126	1.16	.63	58	4
B 681a	80	139	1.22	.74	60	4
B 927a	82	145	1.19	.60	50	5
B1386 b	87	127	1.26	.72	56	Not studied
B1391 b	87	102	1.04	.58	57	Not studied
Average 118			Average 72			
B 609 b	140	139	1.37	1.61	117	4
9211 b	100	118	....	....	...	4
9471 b	125	121	....	....	...	4
B 649 b	125	155	1.43	.82	58	4
B 352a	100	130	1.09	.55	50	5
B 644 b	135	136	1.36	.58	43	5
B 926a	100	160	1.36	.63	46	5
B 926 b	150	143	1.41	.61	44	5
9166 b	105	101	....	....	...	5
9172 b	105	126	....	....	...	5
9470 b	100	100	....	....	...	5
9472 b	100	100	....	....	...	5
Average 128			Average 60			

the weights and histological condition of the testes studied. The testes are grouped into six classes, representing those from rats fed the basal ration for periods of 12 to 40, 50 to 55, 60 to 65, 70 to 75, 80 to 90, and 100 to 150 days, and are listed in the order of their degree of degeneration. Table 2 gives this data in a more condensed form.

It will be readily observed from the two tables that rats fed the basal ration for identical periods show considerable variation in the degree of testicular degeneration attained. This must be attributed to a hereditary difference of the different rats in their reaction to the dietary treatment, for there is absolutely no correlation between the growth curves of similarly treated rats and their degree of testicular degeneration. For example, rats B 255 and B 251, fed the basal ration for eighty-six days, were 121 per cent and 120 per cent normal by weight, but had testes in stages 1 and 5, respectively. Two other rats of this group give a more striking example. Rat B 10, after ninety days, was 114 per cent normal by weight, with a normal testis; while rat B 927, after eighty-two days, was 145 per cent normal by weight, with a testis in stage 5 of degeneration. The latter may be a rather exceptional case, but it serves to show how impossible it is to predict the testicular condition of different rats after any period of dietary treatment between fifty and one hundred days.

However, it is possible to ascertain the histological condition of the testis of similarly treated litter-mates, to a fairly close degree, by the removal and examination of a testis of one of the brothers. This is more accurate if the growth curves of the brothers are approximately the same, for such animals show practically the same testicular condition. Compare rats B 607 and B 609 (sixty-day period); B 577 and B 579 (sixty-five-day period); B 245, B 246, and B 247 (seventy-day period); 9469, 9471, and 9472 (seventy-five-day period); B 255, B 256, and B 257 (eighty-six-day period), and B 251, B 252, and B 253 (eighty-six-day period).

In the fifty-to-sixty-five-day group, eleven of the sixteen testes were in early stages of degeneration, indicating that the degenerative process must have been initiated in these testes somewhat earlier than fifty days—probably nearer the forty-fifth day—of dietary treatment. In the seventy-to-seventy-five-day group, four out of eleven were normal; while after eighty to ninety days only one out of fourteen rats had a normal testis, and this is a rather exceptional animal. Therefore, rats fed the basal ration after weaning may begin to show degenerative changes in the testes at any time within the forty-five to ninety days after feeding is begun, but the process is usually initiated between the fiftieth and sixty-fifth days, since seventeen out of the twenty-one testes exhibiting stage 1 lie within this period of dietary treatment. After one hundred days, degeneration of the testis is practically complete in all animals (compare table 2).

TABLE 2

To show the degree of testicular degeneration found in rats fed the basal ration for twelve to one hundred and fifty days after weaning

PERIOD OF DIETARY TREATMENT	NUMBER OF TESTES STUDIED	NUMBER OF NORMAL TESTES	NUMBER OF TESTES AT				
			Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Days							
12— 40	4	4 (100%)	....	.....	....	....	....
50— 55	16	5 (31%)	9 (56%)	.....	2 (13%)	....	....
60— 65	15	6 (40%)	8 (53%)	.....	....	1 (7%)	....
70— 75	11	4 (36%)	....	.....	1 (9%)	6 (55%)	....
80— 90	14	1 (7%)	4 (29%)	.....	1 (7%)	7 (50%)	1 (7%)
100—150	12	....	....	.....	....	4 (33%)	8 (67%)
Totals	72	20	21	0	4	18	9

After fifty days' dietary treatment, the left testis of rat 9166 was removed, studied histologically, and found to be in stage 1. Fifty days later, the right testis was completely degenerate. Another case, rat 9469, was operated upon after forty days on the diet and the testis removed was normal; thirty-five days later, the other testis showed stages 4 and 5. The process of degeneration from a normal to this advanced condition must have taken place within the thirty-five-day interval between operation and autopsy. A number of other cases also indicate that animals which show a slight degeneration of the testis removed at operation invariably show the condition of stage 5 in the other testis about fifty days later. From the above data it would appear that the process of degeneration, once initiated, passes through to the final condition of stage 5 in from thirty-five to fifty days.

It will be observed (tables 1 and 2) that no testis of this series was listed as being in stage 2 of degeneration. This stage, which is of relatively short duration, is rarely found in the majority of tubules of a testis, and none of the testes of this series happened to show this condition. However, those testes in which the majority of tubules are at stage 3 often show stage 2 in many of the other tubules.

TABLE 3

To show body weight and testis weight of rats fed the basal ration for twelve to one hundred and fifty days after weaning

PERIOD OF DIETARY TREATMENT	NUMBER OF RATS USED	RATS PER CENT NORMAL BY WEIGHT (AVERAGE)	TESTES—NUMBER WEIGHED	TESTES—NORMAL WEIGHT (AVERAGE)	TESTES—ACTUAL WEIGHT (AVERAGE)	TESTES—PER CENT NORMAL BY WEIGHT
Days				*Grams*	*Grams*	
12— 40	4	148	...	....	....	...
50— 55	16	126	8	1.96	2.12	108
60— 65	15	122	14	1.99	1.97	99
70— 75	13	118	9	2.13	1.61	75
80— 90	16	118	14	2.25	1.62	72
100—150	12	128	5	2.67	1.28	44

All the rats used in this and later series were healthy and vigorous, and at autopsy all the organs, except the testes, were in a perfectly normal condition. There is a very marked decrease in the weight of the testes as degeneration proceeds, due to sloughing and removal of the germ cells from the seminal epithelium. When degeneration is complete, the testis weight is approximately one-half the normal weight for rats of the same body weight (compare table 3).

B. Maintenance on the basal ration after the attainment of sexual maturity

The purpose of this series of experiments was to determine whether sexually mature rats, when maintained on the basal ration, would exhibit testicular degeneration similar to that found in rats fed the diet from weaning, and, if so, to determine the approximate time at which degeneration is initiated and the time required for its completion.

Twenty-eight sexually mature male rats, ranging in age from fifty-eight to one hundred and ten days, were fed the basal ration for periods of 25, 50, 75, 85, and 100 days. Six rats had the left testis removed by operation before starting on the basal ration, giving normal control testes of 58, 60, 75, 90, 100, and 115 days, respectively. Twenty-one rats were maintained on the ration for 25, 50, and 75 days before operation and removal of the left testis, and then continued on the same ration for periods of 10, 25, 50, 75, and 100 days, before autopsy and removal of the right testis. One rat (B 1366) was killed without operation.

A comparison of the histology of the two testes of the same rat then served to indicate the changes, if any, taking place in the interval between operation and autopsy. In addition, the operative procedure gave a total of fifty-five testes (six normal and forty-nine at different stages of dietary treatment) from the twenty-eight rats used. Two normal control rats (9327 and 9218) were also used, each from a group of four litter mates, three of which were experimental animals. These two rats were operated upon at fifty-eight and one hundred and fifty days of age, and autopsied at 125 and 200 days, respectively, giving four more normal testes of 58, 125, 150, and 200 days.

The size, scrotal position, and general macroscopical conditions (such as softness, and watery condition when cut) of each testis were carefully noted both at operation and autopsy, but no weighings were made. The body weights (table 4) and growth curves of these rats were usually normal or

TABLE 4

To show the testicular condition of sexually mature normal male rats fed the basal ration for from twenty-five to one hundred days

NUMBER OF TESTIS[1]	INITIAL AGE OF RAT	PERIOD ON THE BASAL RATION	RAT PER CENT NORMAL BY WEIGHT	TESTIS—STAGE OF DEGENERATION	TREATMENT AND CONDITION OF OTHER TESTIS OF SAME RAT (B.R.=basic ration)		
	Days	*Days*				*Days*	
9497a	60	25	134	Normal	B.R.	75	Normal
9274a	88	25	113	Normal	B.R.	75	Normal
9272a	90	25	107	Normal	B.R.	100	Stage 1
9315a	90	25	144	Normal	B.R.	75	Stage 1
9216a	105	25	111	Normal	B.R.	75	Normal
Average			122				
9515a	58	50	121	Normal	B.R.	100	Stage 1
9341a	58	50	96	1 (sl)	B.R.	100	Stage 5
9329a	55	50	102	Normal	B.R.	75	Stage 3
9495 b	60	50	109	Normal	Norm.	60	Normal
9498a	60	50	97	Normal	B.R.	100	Stage 5
9276a	88	50	110	Normal	B.R.	100	Stage 1
9314 b	90	50	127	Normal	Norm.	90	Normal
9273a	90	50	117	Normal	B.R.	75	Normal
9318a	90	50	116	Normal	B.R.	100	Stage 5
9153a	110	50	104	Normal	B.R.	100	Normal
9212 b	105	50	132	Normal	Norm.	105	Normal
9222a	105	50	116	Normal	B.R.	100	Stage 2
Average			112				
9518a	50	75	112	Normal	B.R.	125	Stage 1
9328a	55	75	111	4	B.R.	100	Stage 5
9329 b	55	75	101	3	B.R.	50	Normal
9514 b	58	75	106	Normal	Norm.	58	Normal
B1366ab	58	75	100	*	No operation		
B1357a	58	75	125	*	B.R.	85	*
b	58	85	123	*	B.R.	75	*
B1325a	60	75	95	*	B.R.	85	*
b	60	85	93	*	B.R.	75	*
B1293a	60	75	92	*	B.R.	85	*
b	60	85	91	*	B.R.	75	*
9497 b	60	75	136	Normal	B.R.	25	Normal
9274 b	88	75	115	Normal	B.R.	25	Normal
9273 b	90	75	111	Normal	B.R.	50	Normal
9315 b	90	75	123	1 (sl)	B.R.	25	Normal
9216 b	105	75	108	Normal	B.R.	25	Normal
9217a	105	82	109	Normal?	B.R.	100	Stage 1
9158a	110	75	130	Normal	B.R.	180	Stage 5
Average			110				
9328 b	55	100	115	5	B.R.	75	Stage 4
9341 b	58	100	80	5	B.R.	50	Stage 1 (sl)
9515 b	58	100	120	1	B.R.	50	Normal
9498 b	60	100	85	5	B.R.	50	Normal
9276 b	88	100	104	1	B.R.	50	Normal
9272 b	90	100	87	1 (sl)	B.R.	25	Normal
9318 b	90	100	106	5	B.R.	50	Normal
9199 b	100	100	118	5	Control	100	Normal
9217 b	105	100	96	2	B.R.	82	Normal
9222 b	105	100	97	2	B.R.	50	Normal
9153 b	110	100	117	Normal	B.R.	50	Normal
Average			102				

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[1] The testis number, in each case, corresponds to the number of the rat from which it was taken.

a, after the number, indicates the testis removed at operation.

b, after the number, indicates the testis removed at autopsy.

ab, after the number, indicates that both testes were removed at the same time without previous operation.

* Testis not yet studied histologically.

well above normal, although a few were slightly below in weight at operation and autopsy, but this is well within the variation found in normal healthy rats.

At autopsy, all the animals were found to be in a perfectly healthy condition with the exception of two rats (9218 and 9222) which showed slight lung infection, but this was not serious. All the internal organs except the testes appeared normal.

Reference to tables 4 and 5 shows that twenty-five days' dietary treatment is not sufficient to induce testicular degeneration, while, after fifty days, only one out of twelve testes showed any indications of degeneration, and about half the tubules in this testis were still normal. After seventy-five days, three out of eleven testes studied were degenerate, and one of those only slightly so, while after one hundred days, only one out of eleven was normal, five were in early stages, and five were completely degenerate. Thus, testicular degeneration is usually not initiated much before seventy-five days, nor much later than ninety or one hundred days, in sexually mature rats, as compared with fifty to sixty-five days in the case of rats fed the basal ration from weaning.

TABLE 5

To show the degree of testicular degeneration in sexually mature rats maintained on the basal ration for periods of twenty-five, fifty, seventy-five, and one hundred days

PERIOD OF DIETARY TREATMENT	NUMBER OF TESTES STUDIED	NUMBER OF TESTES AT					
		Normal	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Days							
25	5	5	...	...	...	...	...
50	12	11	1	...	...	...	...
75	11	8	1	...	1	1	...
100	11	1	3	2	...	...	5

The time required to complete degeneration is approximately the same in both series, i.e., thirty-five to fifty days. Two cases will be sufficient to illustrate this. The left testes of rats 9498 and 9318, removed after fifty days' dietary treatment, were perfectly normal. After fifty more days, the right testes of both were completely degenerate. Assuming that the degeneration began immediately after the removal of the left testis, and that the process was just completed at autopsy, the time required would have been fifty days. While the maximum time may lie not far within this fifty-day period, the minimum time required for the process is somewhat less, and corresponds closely with the results of the previous series (p. 185).

It may be mentioned at this time that, from the study of almost one hundred testes from rats in which the other testis had been removed by operation from twenty-five to one hundred days previously, there is absolutely no evidence for the assumption that the rate of degeneration or time at which degeneration sets in is any different when one testis is removed than if it were present. This is in opposition to the view held by Kuntz ('19), after his study of testis degeneration in dogs.

ATTEMPTED CURE OF THE DIETARY STERILITY

After finding the definite testicular degeneration in rats fed the basal ration, it was decided to see whether such degenerate testes could be brought back to a normal condition by putting the animals on the stock-food diet of dog biscuit and carrots (supplied twice a week) used for the stock or breeding colony or by adding specific food substances to the basal ration of known composition.

About the time these experiments were being inaugurated, the preliminary paper of Evans and Bishop ('22) appeared, postulating the existence of a ''hitherto unrecognized dietary factor necessary for reproduction'' which they termed vitamine X or the reproductive vitamine, and which they have now classed as vitamine E (Evans, '25; Evans and Burr, '25). This factor, which proved very efficient in preventing and curing the disorder in female rats reared on 'purified food' diets, was found to be abundant in certain natural foodstuffs, especially fresh green leaves of lettuce and whole-wheat germ.

Although the disorder in the female was of an entirely different nature from that of the male, as has been mentioned (p. 161), it was decided to determine the ability of lettuce to cure the testicular degeneration in the males, and to see whether this condition is also due to lack of the specific factor, vitamine E, in the basal ration.

Since different rats vary greatly in the degree of testicular degeneration, it was impossible to determine the histological condition of the testes after any definite period of treatment. However, in most cases sufficient time was allowed to insure some degree of degeneration, which was determined by study of the testis taken at operation. This was compared with the condition found in the other testis after different periods of treatment on the stock food or with the lettuce addition.

A. By return to stock-food diet

The results of this series are not very conclusive. Eight male rats were fed the basal ration from weaning until operation (fifty to eighty days), when one testis was removed for study. All these testes showed some stage of degeneration and mostly stage 1.

After operation the rats were fed the ordinary stock-food diet for periods of twenty-five to seventy-five days, before autopsy and study of the remaining testes. In all cases the second testis was much more degenerate than the first. It is rather unfortunate that no rats were kept on the stock ration for much longer periods of time (say 150 to 250 days). However, in view of the results obtained by lettuce feeding (described below), it is rather doubtful if positive improvement would have been noticeable in the second testis, even after ten or twelve months' feeding on stock food, since this diet probably does not contain a very large excess of vitamine E over the normal requirements for reproduction.

B. By addition of lettuce to the basal ration

This series consists of thirty-six male rats which were placed on the basal ration immediately after weaning. After periods of from fifty to one hundred days' maintenance on the ration, the left testis was removed by operation (unilateral castration) and studied histologically, to determine the degree of degeneration at that time. The rats were then placed on the same ration plus 40 grams of fresh green lettuce daily for from forty-five to one hundred and fifty days before autopsy, when the right testis was removed for histological study. Four control rats, litter mates of a number of the above, were treated in the same manner except that no lettuce addition was given after operation. A comparison of the two testes of the same rat, before and after the lettuce treatment, would indicate the beneficial effect of the lettuce, if any, as compared with what would have happened had no lettuce addition been given (as shown by the controls). Ten rats from another series served as additional controls.

The growth curves and body weights (table 6) of practically all these rats, up to the time of operation, showed the usual excellence so characteristic of rats reared on the basal ration. Three of these rats had a body weight at operation slightly below normal figures given for rats of their age, but they were perfectly normal and healthy. Such a slight difference in body weight is easily within the range of normal variation and would not necessarily be any indication of a lower vitality or other abnormal condition of the rat.

However, after operation and addition of 40 grams of lettuce daily to the basal ration, all the rats showed a phenomenal increase in body weight and excellent growth curves. Table 6 serves to give some idea of the rapid increase in body weight of such rats during the various periods of lettuce feeding, as compared with the increase for the same interval in normal rats of the same age.

That this rapid increase in body weight after operation is due to the effect of the lettuce, rather than to the effect of the unilateral castration procedure, can readily be shown. It is known that complete castration may result in a tendency for accumulation of large fat deposits in some animals and would naturally result in increased body weight. Whether removal of one testis only would have a similar but less marked effect is doubtful, but it is certain that such a very marked increase, as obtained in the rats given lettuce after operation, could not be due to such a cause. A typical case will be sufficient to show this, as well as a comparison of the growth rate of rats with and without the lettuce addition after operation.

Chart 2 shows the growth curves of two litter mates which were fed the basal ration for sixty days before operation. After removing one testis from each rat, one (B 644) was continued on the basal ration alone, while the other (B 648) was fed the lettuce addition. Rat B 644, although fed the basal ration throughout, showed a curve well above normal, as is true of practically all rats fed this ration. But rat B 648 shows a remarkable increase in growth over B 644 in the same period of time, as a result of the lettuce addition.

TABLE 6

To show rapid increase in body weight of rats after adding 40 grams of fresh lettuce to basal ration

PERIOD OF LETTUCE TREATMENT AFTER OPERATION	NUMBER OF RATS USED	AVERAGE WEIGHT OF RAT AT OPERATION	AVERAGE WEIGHT OF RAT AT AUTOPSY	AVERAGE INCREASE DURING LETTUCE TREATMENT	NORMAL INCREASE FOR SAME PERIOD	PER CENT INCREASE OVER NORMAL
Days						
45— 60	5	195 (161)[1]	294 (217)	99	56	77
75— 80	12	200 (180)	353 (218)	153	73	210
100	12	185 (141)	377 (238)	192	97	200
125—150	7	203 (177)	389 (265)	186	88	211

[1] Figures enclosed in brackets represent the normal body weight of rats at this age.

The growth-rate comparison is perhaps better shown in the two litter mates, B 924 and B 926 (chart 2). After fifty days' maintenance on the basal ration, both had approximately the same body weight. Rat B 924 was then given 40 grams of lettuce daily in addition to the regular ration. At autopsy, 100 days later, B 926 weighed 334 grams, while B 924 had reached a weight of 475 grams and was still gaining rapidly. Rat B 925, another brother, was treated the same as B 924, but was autopsied after fifty days of lettuce feeding. Although weighing 42 grams less than B 926 at the fifty-day period, B 925 surpassed the latter by 26 grams during the fifty days of lettuce feeding.

The data for this series are summarized in table 7. The periods of dietary treatment and histological condition of the testes at the termination of these periods are given for each rat. The rats have been listed in groups representing those given the lettuce treatment for periods of 45 to 60, 75 to 80, 100, and 125 to 150 days, and will be described in this order.

Forty-five to sixty days. Two rats of this group will be described somewhat in detail, since they afford good examples of the conditions met with in this series of experiments. Rat B 925 (chart 2) showed a normal histological condition of the testis removed at operation, after fifty days on the basal ration, and after fifty days more on the basal ration plus the lettuce addition the second testis was still perfectly normal. During the fifty-day period the body weight of the rat increased from 138 to 336 grams, while the figures given by Donaldson for body weights for the same age periods are 110 and 193 grams, respectively—an increase of nearly two and one-half times that of normal rats for the same period. Both testes were 6 per cent above normal by weight, showing an increase exactly in proportion to the body weight. Rat B 926 (table 1), litter-mate control of B 925, had one testis removed by operation after maintenance on the basal ration for the entire 100 days without the lettuce addition. The body weight and testes weight at this time were 309 grams and 0.63 gram, respectively, as compared with 336 grams and 1.5 grams, the corresponding figures for B 925. It will be seen that B 926 has suffered a marked decrease in testes weight, and histological study shows the testis to be at stage 4 of degeneration (fig. 5). Since no evidence has been found to indicate that rat 926 would have shown a different degree of histological degeneration had one testis been removed after

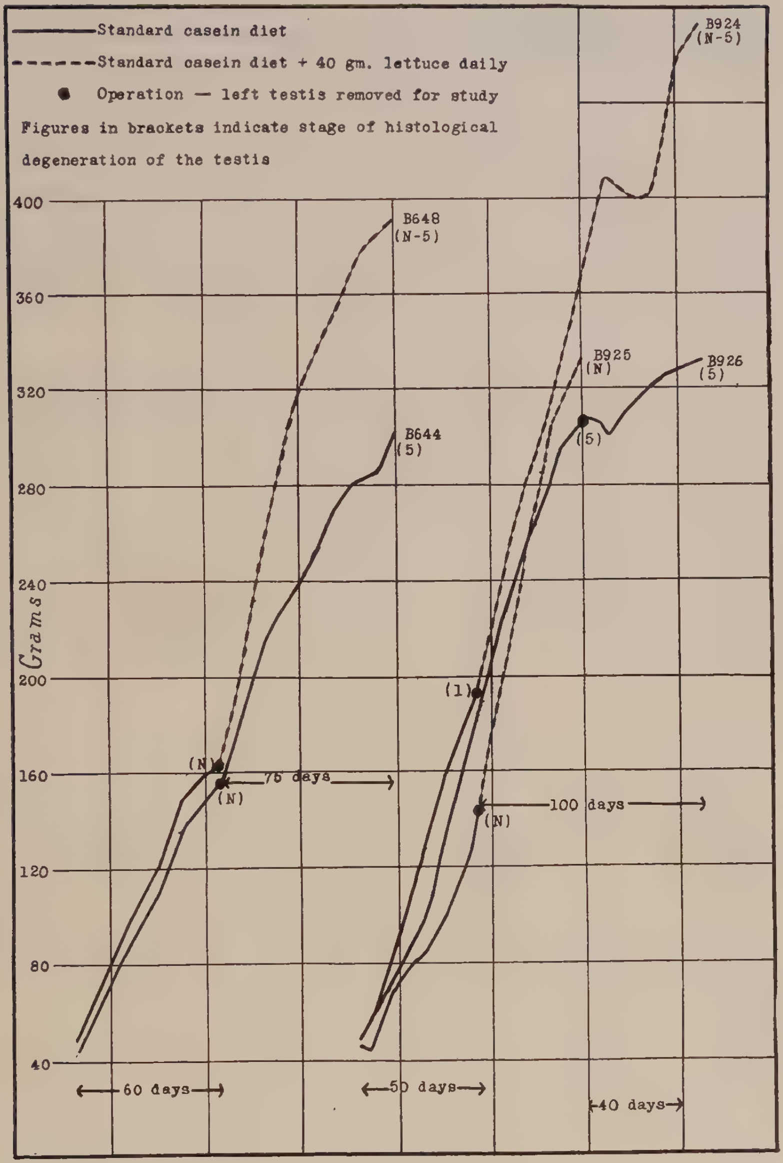

Chart 2 Showing the beneficial effects of supplementing the basal ration with daily additions of 40 grams of fresh lettuce upon the growth rate and testicular conditions of male rats after previous feeding on the basal ration alone.

TABLE 7

To show 'partial prevention' of the testicular degeneration by the daily addition of 40 grams of lettuce to the basal ration

NO. OF THE RAT	PERIOD ON BASAL RATION AFTER WEANING		HISTOLOGICAL CONDITION OF TESTIS		PREVENTION
	To operation	Plus lettuce, to autopsy	At operation	At autopsy	
	Days	*Days*			
B 925	50	50	Normal	Normal	No degeneration
B 245	70	60	Normal?	N–5	Partial
B 255	86	45	Stage 1	Stage 5	....
B 251	86	45	Stage 4	Stage 5	....
B 696	75	50	Stage 4	Stage 5	....
B 10	93	75	Normal	Normal	No degeneration
B 53	75	75	Normal	Normal	No degeneration
B 642	60	80	Normal	Normal	No degeneration
B 639	65	75	Normal	N–5	Partial
B 648	60	75	Normal	N–5	Partial
B 679	60	75	Normal	N–5	Partial
B 607	60	80	Normal	N–5	Partial
9987	87	80	Stage 1	Stage 5	....
B 30	75	75	Stage 3	Stage 5	....
B 617	80	75	Stage 4	Stage 5	....
B 625	70	75	Stage 4	Stage 5	....
B 681	80	75	Stage 4	Stage 5	....
B 812	50	100	Normal	Stage 5	....
B 649	60	100	Stage 1	Normal	Recovery?
B 526	55	100	Stage 1	N–5	Partial
B 859	50	100	Stage 1	N–5	Partial
B 579	65	100	Stage 1	N–5	Partial
B 924	50	100	Stage 1	N–5	Partial
B 540	55	100	Stage 1	Stage 5	....
B 577	65	100	Stage 1	Stage 5	....
B 723	60	100	Stage 1	Stage 5	....
B 614	60	100	Stage 4	4–5	Partial?
B 897	50	100	Stage 3	Stage 5	....
B 352	100	100	Stage 4	Stage 5	....
B 246	70	125	Normal	N–5	Partial
B 256	86	125	Stage 1	Stage 5	....
B 257	86	150	Stage 1	1–5	Partial
B 252	86	125	1–4	N–5	Partial
B 253	86	150	1–4	N–5	Partial
B 260	86	150	1–3	1–5	Partial
B 927	82	140	Stage 5	Stage 5	....

fifty days, as in the case of B 925, and since litter mates differ very slightly in their degree of testicular degeneration under the same experimental conditions, provided there is no subnormal growth of the rats, we can assume that the testis of B 926, after 100 days on the basal ration, indicates what would have been the condition of the second testis of B 925 if the lettuce had not been supplied during the last fifty days. It is reasonable to conclude, then, that the lettuce has supplied something which has prevented the advanced condition of degeneration which otherwise would have occurred.

Rat B 245 at operation had a testis which might almost be classed as normal, although the tubules seemed slightly immature, and several tubules were found exhibiting a very slight degree of spermatozoa degeneration. After sixty days' treatment with the lettuce addition, the second testis showed the majority of tubules at stage 5 of degeneration, but about 8 per cent or 10 per cent of the tubules were normal, or practically so, most of them producing spermatozoa in large numbers. These large normal tubules offer a striking contrast when compared with the small degenerate tubules around them, which are from one-half to one-quarter the size of the former. Undoubtedly, all the tubules would have undergone degeneration to stage 5 had not the lettuce been added. It is true that in the tests of many rats fed the basal ration alone, there is often a rather wide variation in the degree of degeneration of the different tubules. For instance, a testis listed as being at stage 3, with most of the tubules showing the giant-cell condition, may exhibit stages 2, 4, and, rarely, 1 or 5, but these are always very small in number. However, in all cases where the majority of tubules were at stage 5, the other tubules in that testis seldom showed a condition less advanced than stage 4 or a late condition of stage 3.

Therefore, in the above rat, B 245, we have something entirely different from any condition previously encountered. The lettuce addition has certainly had some beneficial effect on the testicular condition, but it is impossible to say how long a period elapsed, after the change of diet, before the beneficial effect of the lettuce asserted itself. Consequently, it is difficult to tell the exact histological conditions of the various tubules at that particular time. The results of Evans and Bishop ('23 a), showing restoration of fertility in female rats supplied with the lettuce addition about the beginning of the gestation period of three weeks, indicates that the lettuce produces its beneficial effect in a relatively short time. Rat B 925 also indicates a rather rapid effect in totally preventing the testicular degeneration which, otherwise, would have soon been inaugurated by continuance on the basic ration alone. This is purely a case of prevention, not cure, of the degenerative condition. The case of B 245, too, indicates a prevention of degeneration in those tubules which were still normal, or nearly so, at the time when the beneficial effect became actual, while those tubules in slight and more advanced stages of degeneration continued to the extreme condition, regardless of the lettuce treatment.

In the remaining three rats testicular degeneration had already progressed too far and, as a result, the second testis after the lettuce treatment showed complete degeneration identical with that which would have been attained, in a similar period, on the basal ration alone.

The histological evidences of the disorder are probably but poor indications of a much deeper lying physiological upset involving the entire germinal epithelium. From these, and other cases to be described later, there appears to be a physiological 'threshold of sterility,' as it might be called, beyond which the condition of the germinal epithelium cannot be improved by the lettuce supplement. Just where this may lie in relation to the histological picture is impossible to determine closely, but probably somewhere between the normal condition and the early stage of spermatozoa degeneration designated as stage 1. For this reason, such conditions as seen in rat B 245, and in other rats described below, can scarcely be referred to as showing 'recovery,' but are more correctly conditions of what might be termed 'partial degeneration' or 'partial prevention.' For convenience in describing the results of this series of experiments, the term 'partial prevention' will be used to indicate testicular conditions showing normal or seminormal seminiferous tubules (usually in the minority) associated with a very advanced or complete degeneration in the remaining tubules of the testis.

With these few points in mind, an examination of the results of the other groups of this series will be greatly simplified.

Seventy-five to eighty days. Twelve rats, maintained for various periods on the basal ration after weaning before removal of one testis by operation, were given lettuce, in addition to the basal ration, for seventy-five to eighty days. Three of these (B 10, B 53, and B 642) showed a normal condition of the testes both at operation and autopsy, similar to rat B 925 of the previous group. All show an increase in testis weight since operation, about in proportion to the increase in body weight.

Four other rats (B 639, B 648, B 679, and B 607) each showed a normal testis at operation and the condition of 'partial degeneration' in the testis at autopsy. The first three had about 24 per cent and the latter about 15 per cent of the tubules of the second testis in an extreme stage of degeneration, while the rest of the tubules were perfectly normal and in active spermatogenesis. The epididymis in all cases contained large quantities of normal spermatozoa. The testes removed at autopsy were not so large, in proportion to the body weight of the rats, as were those taken at operation, but they are still approximately normal in weight.

Of the remaining five rats, one showed stage 1, one stage 3, and three stage 4 in the testes taken at operation. After seventy-five to eighty-five days' lettuce treatment, the second testes of all these rats showed complete degeneration to stage 5. Apparently no normal tubules were present in these testes at the time when the lettuce could show its effect, and degeneration continued as if no lettuce had been added. The testicular condition of one of these rats (B 681) at the time of operation, before lettuce feeding, is shown in figure 6 A.

The testes of these five rats at operation averaged 77 per cent normal by weight, and at autopsy 52 per cent normal by weight, due to the loss of degenerated germ cells. The actual weights of the testes were practically the same at autopsy as at operation, in spite of the great increase in body weight of the rats from an average of 212 grams to 345 grams.

One hundred days. This group includes twelve rats treated like those of the previous group, except for the longer retention on the lettuce addition. Two of these are of particular interest.

Rat B 812 shows a perfectly normal condition in one part of the testis, removed at operation, while another part showed marked spermatozoa degeneration (stage 1) in many of the tubules. In the preparation of the testis for histological study, as is true of practically all the testes studied in this investigation, the testis was snipped into small pieces, those of the anterior half being fixed in Bouin or Allen's B 15 fixative and those of the posterior half in Zenker or Helly's formol-Zenker mixture. Then at least two slides were made from material from the two fixations, representing different portions of the same testis. Almost invariably the two halves have shown identically the same histological picture. In this rat the Bouin preparations showed a normal condition, but the Helly material was in stage 1 of degeneration. The second testis of this rat, after 100 days' lettuce treatment, showed complete degeneration in all the tubules. It may be that either the physiologically abnormal changes in the histologically normal tubules of the testis taken at operation were too far advanced for the lettuce to have its beneficial effect in preventing degeneration of similar tubules in the second testis or the lettuce was unusually slow in asserting its effect on the latter testis.

The other rat of particular interest is B 649. The first testis of this rat showed distinct sperm degeneration, while the second testis, after 100 days' lettuce treatment, showed a normal condition. This would seem to indicate an ability of the seminiferous tubules to return to a normal condition, with lettuce treatment, after they had reached stage 1 of degeneration. This does not agree with the results from eleven other rats in this series, which showed testes at stage 1 at the time of operation, for the second testes of these rats, after lettuce treatment, showed either 'partial prevention' or complete degeneration to stage 5. Rat 648 of the previous group, litter mate of this rat, had a normal testis at operation and after seventy-five days' lettuce treatment, the other testis showed 'partial prevention.' The testis weights of B 648 and B 649 at operation were 1.06 and 0.77 grams, respectively, and 1.44 and 1.55 grams at autopsy. Although B 648 shows a somewhat better growth curve throughout the experiment, B 649 certainly does show a more marked improvement in the testis weight than its litter mate, which cannot be entirely accounted for by the additional twenty-five days' treatment with lettuce. At operation, note was made that the testis of B 649 was 'soft, but fairly normal.' This, together with the slightly subnormal weight of the testis (90 per cent normal), would naturally indicate a slightly abnormal histological condition, as microscopical studies showed.

From the above facts it would appear that addition of lettuce to the basal ration for a period of 100 days can restore to a normal condition a testis in which many of the tubules previously showed stage 1 of degeneration. But, since this is so out of accord with the results obtained from all the other cases studied in the entire series, the data do not seem adequate to warrant such a statement.

Of the seven other rats of this group which showed stage 1 in the testis removed at operation, four (B 526, B 579, B 859, and B 924) exhibited 'partial prevention,' and three (B 540, B 577, and B 723) showed complete degeneration of the second testis removed after 100 days' lettuce treatment.

Rat B 526 exhibits the greatest degree of partial prevention in the second testis, with about thirty normal tubules in the entire cross-sections of the organ; the remaining tubules all show complete degeneration and are about one-fourth the size of the former. The epididymis contains large quantities of normal spermatozoa, indicating that the tubules which have retained their normal condition are in an active state of spermatogenesis. Rat B 859 showed about a dozen normal and seminormal tubules, but spermatogenesis is not as rapid as in the above rat, and the ducts of the epididymis contain large masses of loose degenerating cells in which are entangled small numbers of spermatozoa. A smear slide prepared from the epididymis, before fixation, showed a considerable number of spermatozoa, many of which showed greatly swollen sperm heads and other abnormal conditions of the chromatin so characteristic of stage 1.

Rat B 579 showed a very slight 'partial prevention,' with only two or three tubules, out of several hundred in a cross-section of the testis showing a normal condition. The ducts of the epididymis showed numerous spermatozoa, many of which are very abnormal, intermingled with masses of loose degenerating cells.

Rat B 924 shows only a very slight type of 'partial prevention,' for no normal tubules are present, but from 15 to 20 per cent of the tubules are in a semidegenerate condition, still containing spermatogonia, spermatocytes, and a few immature spermatozoa in a rather sickly condition. The epididymis of this testis shows large masses of degenerate cells in the ducts and a very few abnormal spermatozoa. One naturally wonders whether the latter represent spermatozoa which are being produced by the few seminormal tubules, and later undergo degeneration in the epididymis, or whether, like the loose cells in the ducts of the epididymis, they are the previous products of the tubules which at this time show complete degeneration. A study of the epididymides of the second testes of the three other rats, whose first testes were also at stage 1 before the beginning of lettuce treatment and whose second testes gave no evidence of 'partial prevention,' shows the same type of loose cell masses in the ducts, but no sign of normal or degenerate spermatozoa. This and similar evidence from other cases studied would support the former alternative.

The remaining three rats (B 897, B 614, and B 352) showed stages 3 and 4 in the first testes and complete degeneration in the second testes, after 100 days on the lettuce addition. The tubules of these second testes, at the time of operation, had become too degenerate to be relieved by the lettuce treatment. Rat B 614 showed stage 3 in the majority of tubules of the first testis. However, a number showed a lesser degree of degeneration, a few of them containing sperm heads and degenerate sperm, but no mature spermatozoa could be found. After 100 days' lettuce treatment, the second testis showed about 30 per cent of the tubules which had retained considerable of the germinal epithelium, although the latter is far from normal and the size of these tubules has been reduced to practically that of the very degenerate ones. Apparently, the lettuce began its beneficial effect in time to prevent complete loss of the germinal epithelium of a number of the tubules not so severely injured as the others. It is possible that prolonged lettuce treatment might have caused a return of some of these tubules to a normal condition, but 100 days is certainly not sufficient to do so. After close examination, it was impossible to detect any mitoses in the cells of these semidegenerate tubules and, judging from the general appearance as well, the destructive and preventative forces involved had practically reached a balance.

One hundred and twenty-five to one hundred and fifty days. In this group three rats had the lettuce addition for 125 days, one for 140 days, and three for 150 days following operation and removal of one testis, after maintenance on the basal ration for from seventy to eighty-six days.

Rat B 246 showed a normal testis at operation and, after 125 days' lettuce treatment, the second testis had from 65 per cent to 75 per cent of the tubules at stage 5, while the rest were either normal and in active spermatogenesis or seminormal with immature spermatozoa and greatly reduced in size. A few cases of mitotic divisions were found in these seminormal tubules.

Rats B 256 and B 257 are litter mates which had similar operative and dietary treatment, except that the latter was held on the lettuce addition twenty-five days longer. The testes of both at operation showed sperm degeneration in practically every tubule, but B 256 was slightly further advanced that B 257. After 125 days' lettuce treatment, the other testis of B 256 showed complete degeneration. After 150 days of similar treatment, the other testis of B 257 showed 'partial prevention,' about ten tubules being in a seminormal condition and showing a few mitotic cell divisions in the basal layer.

Rats B 252 and B 253 were litter mates also, treated identically with the two rats described above. The first testis of each of these showed rather advanced degeneration in a few tubules, but a considerable number in a seminormal condition. The second testes, after lettuce treatment, showed that about one-fifth of these seminormal tubules had been prevented from undergoing degeneration, and in B 252 a few were producing normal spermatozoa, but not very rapidly.

B 260, in the testis taken at operation, showed tubules in stages from 1 to 3, but no normal ones were present. After 150 days' lettuce treatment, about 90 per cent of the tubules were completely degenerate, and the remainder, although greatly reduced in size, had a seminormal epithelium containing various stages of spermatogenesis up to poorly developed spermatozoa heads. Although these tubules have not returned to a normal condition after 150 days' lettuce treatment, it is not impossible that such might occur after considerably longer periods of lettuce feeding. This testis did show a marked increase in weight, but it was extremely turgid with the watery fluid produced from the cellular liquefaction and edema of the organ. The pressure resulting from the enclosed fluid may, in itself, prevent return of the seminormal tubules to a normal condition.

We must conclude that, after testicular degeneration has been initiated by feeding the basal ration, the daily addition of 40 grams of fresh lettuce for periods up to 150 days can preserve a normal condition in tubules which otherwise would have degenerated, but cannot restore degenerating tubules to a normal condition.

PREVENTION OF THE DIETARY STERILITY

A. By rearing on basal ration plus lettuce

Three groups of four litter mates each were used in this series. Two animals from each group were fed the basal ration, supplemented with lettuce, throughout the experiment. The other two were either fed the basal ration alone throughout or were given the lettuce supplement after removal of the left testis.

The lettuce addition from the beginning of feeding, in all cases, prevented the testicular degeneration which appeared in the controls deprived of the lettuce. A typical group of four litter mates is shown in chart 3. The lettuce-fed rats, B 693 and B 699, showed an especially rapid rate of growth and normal testes both at operation and autopsy. The other two brothers, on the basal ration for seventy-five days, had very degenerate testes (stage 4) at operation and completely degenerate testes at autopsy, although one of them (B 696) had been given the lettuce supplement during the fifty days since operation. There was a rapid increase in growth in this rat, but the testicular changes were too far advanced to show any evident improvement.

Figures 1 and 5 were drawn from portions of the left testes of rats B 699 and B 696, respectively, showing the striking contrast between the testes of two litter mates, one of which was reared on the basal ration with the lettuce addition for seventy-five days, while the other was deprived of the lettuce for the same period.

Another group of four rats (chart 3) shows a similar condition. Rats B 580 and B 581, fed the lettuce supplement throughout, had perfectly normal testes both at operation (100 days) and at autopsy (165 days). The other two brothers, fed the basal ration alone up to the time of operation (sixty-five days) showed stage 1 of degeneration in the testis removed. After 100 days more feeding, even though the lettuce supplement was added during this period, the remaining testes were completely degenerate.

DISCUSSION

The testicular degeneration

As is well known, the testis is a complex gland, very sensitive to unnatural physiological conditions of the animal body as a whole, as well as to the influences of various external environmental factors. Consequently, the testicular degeneration described in the early part of this paper is not at all new, for biological literature contains many descriptions of such conditions in man and other animals, and those have been attributed to a great variety of factors.

The degeneration due to the effect of x-rays on the testis is well known and has been described by Bergonie and Tribondeau ('04) in rats, Regaud ('10) in the rabbit, guinea-pig, mouse, cat, and rat; Barratt and Arnold ('11) in the rat, and others. The process described by these authors is somewhat different in that they find the spermatogonia to be the first cells affected by the rays, followed by the spermatocytes, spermatids, and lastly the spermatozoa. The reason for this reversed order of degeneration is difficult to explain, unless the rays have a specific effect on the cells undergoing more rapid mitotic division. In general, the cytological and pathological conditions found by these workers are similar to those described here and the end result is the same.

The degenerative changes mentioned by other workers, and ascribed to a great variety of causes, more nearly agree with those described in this paper, but very few authors have described the detailed histological changes involved. The atrophic and degenerative changes in the testis during total and partial inanition, described by Grandis ('89) in pigeons, Loisel ('01) and Poikarov ('13) in the dog, and Siperstein ('21) in the rat, are due to a decrease or total lack of the reproduc-

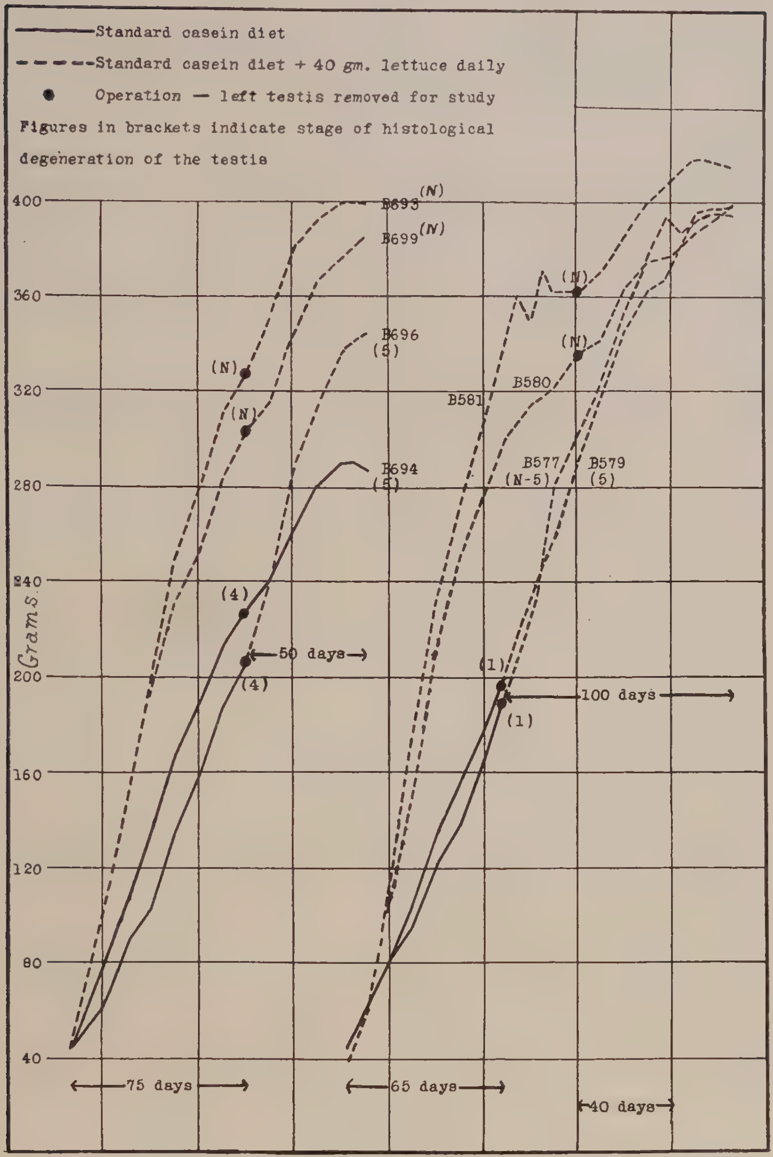

Chart 3 Showing the difference in growth rate and testicular condition of rats fed the basal ration, with and without the addition of 40 grams of fresh lettuce daily.

tive vitamine E, as well as to a poor physiological condition of the body in general, resulting from diminished quantity or lack of other necessary food factors. The work of Siperstein ('21) gives the most complete histological study of such conditions.

Similar changes observed in vitamine deficiency by Funk and Douglas ('14) in pigeons, McCarrison ('19, '20) in pigeons and monkeys, Allen ('19 a) in rats, Dutcher and Wilkins ('21) in cockerels, Meyerstein ('22) and Eckstein ('23) in rats, and by Cordes ('98) and other workers in chronic and wasting diseases, are also due to a greatly lowered physiological condition of the body as a whole, as well as to a specific lack of several vitamines, which would naturally have a marked effect on such a sensitive organ as the testis.

The degeneration ascribed by Paul ('06) and Hewer ('14) to excessive meat feeding is the result of a poor food balance and consequent lack of certain necessary food and mineral substances not present in meat. Similarly, Osborne and Mendel have failed to get reproduction on a diet of 95 per cent meat residue and 5 per cent salts, plus alfalfa (0.4 gram) and yeast (0.2 gram) to supply the water-soluble vitamine B.

Alcoholization over long periods produces testicular degeneration in rats, as found by Bouin and Garnier ('00) and Allen ('19 a). Whether this is due to a specific action of the alcohol on the germinal epithelium or the indirect result of a lowered vitality and other body disturbances, is not known, but the latter seems more probable.

Kuntz ('19) has also described testicular degeneration in the dog after elimination of the sympathetic nerve supply, and Moore ('24) in testis grafts and experimental cryptorchidism and ('24 a) after heat application to the scrotum. These cases here are due to specific effects on the testis itself, and not to a poor condition of the body as a whole.

It is of particular interest that, with the exception of x-rayed testes, the process of degeneration is essentially the same regardless of the cause. However, the histological data given by most workers are rather meager, and in many cases only the more advanced conditions have been observed, due to the small number of animals studied and the greater rapidity of the process in more severe treatment, so that intermediate stages are only occasionally described. Because of the slower rate of degeneration in my material and the large number of rats used, the more detailed stages of the process have become more evident.

Furthermore, in using the standard casein diet (basal ration) of known chemical nature, which affords excellent growth and a very normal healthy condition of the animals, the factors of lowered vitality and general body disturbances are eliminated, and the testicular degeneration must be attributed to a lack of a specific substance or substances in the basal ration. The more exact nature of this lack will be considered after the discussion of a few points of interest in regard to the degenerative process.

Duration of the degenerative process

The time required for completion of the degenerative process depends on the severity of the cause and the relative resistance of the germinal epithelium. It has been shown that in rats fed the basal ration after weaning (p. 185) or after the attainment of sexual maturity (p. 189), the degenerative testicular changes from the normal condition to that of complete degeneration (stage 5) usually occur within an interval of thirty-five to fifty days.

Bouin and Garnier ('00), in the testes of two rats alcoholized daily for eight and one-half and eleven and one-half months, respectively, found practically all stages of degeneration in the different tubules of a single testis, as a result of a differential susceptibility of the various tubules, associated with an extremely long-drawn-out process.

Moore ('24, '26) finds complete disorganization of the germinal epithelium of testes retained in the peritoneal cavity for seven days and complete degeneration after twenty days. The different tubules vary somewhat in their degree of degeneration, but this is not quite so noticeable as in my material.

This process described by Moore, both after abdominal retention and heat application ('24 a), is a relatively rapid process.

The degeneration described in this paper lies intermediate between the two types mentioned above, for about three months is usually necessary to complete the process, although the actual degeneration and removal of the cells requires from thirty-five to fifty days, once the process has been initiated.

Because of the limited number of cases studied by Bouin and Garnier and the very rapid process of Moore, these workers have apparently failed to find the condition of stage 2 of this paper. This condition is not very abundant in my material, due to the short time required for this particular phase of the process, but it is of importance in explaining the origin and nature of the multinucleate giant cells so abundant in stage 3.

The multinucleate giant cells

Barratt and Arnold ('11), Monterosso ('12), and other authors claim to have found giant cells in tubules of normal testes, due to fusion of neighboring cells, but none of the normal control testes used in this study have shown such a condition. Siperstein ('21) finds none in normal testes, and authors describing the normal spermatogenesis of the rat do not mention finding such cells in their material.

However, the giant-cell formation is a relatively frequent phenomenon in testis degeneration, and has been described after aseptic wound healing (Maximow, '99), in seasonal degeneration of testes in hibernating animals (Tandler and Grosz, '11), in experimental cryptorchidism (Moore, '24), in inanition (Siperstein, '21), after alcoholic treatment (Bouin and Garnier, '00), and after x-ray treatment (Barratt and Arnold, '11).

Bouin and Garnier believe that these giant cells are formed by abnormal mitoses of the nuclei within the cell, but no indications of this have been observed in the large number of testes studied in my work. It is possible, of course, that germ cells in a slightly weakened condition, such as alcoholic treatment might produce, would not be capable of completing the process of mitosis, and thus form binucleate or even four-nucleated cells, but such a method could not account for the enormous cells found in my material.

Allen ('19 a) noticed these giant cells during the early stages of degeneration, but offers no explanation of their origin. Eckstein ('23) also observed these, but did not mention their significance.

Moore ('24) describes these, and states that they result from "the fusion of contiguous germ cells." Siperstein ('21) states that they are formed "by a fusion of primary spermatocyte cells, when they begin to degenerate."

The writer has shown (p. 174) that the great majority of the giant cells arise by a coalescing of a varying number of adjoining degenerate spermatids, and (p. 176) that only in later stages, when these have almost disappeared, do the primary spermatocytes fuse to form such structures, and these are of much smaller size and relatively rare.

Maximow ('99), in testicular degeneration of the dog, found enormous giant cells identical with those in my material, and describes their formation from degenerating spermatids in the same manner as I have described it. His figures 20 to 26, 47, 48, and 51 to 57 show these giant cells, containing spermatid nuclei, in all stages of formation. He and Bouin ('97) also find amitotic divisions of degenerating spermatocytes which I have failed to find in my material. Maximow gives an excellent review of the literature and a very complete bibliography of similar work done up to that date.

Wakelin, Barratt and Arnold ('11) observed similar giant cells in x-rayed testes of the rat, and picture two of these (their figs. 24 and 25) containing the vesicular nuclei which I have described as spermatid nuclei,[4] although they considered them altered secondary spermatocytes, but were not sure. They also believe that these giant cells arise by fusion of the degenerate cytoplasm of these cells.

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[4] See footnote 3, p. 171.

The Sertoli syncytium

In the advanced stages of degeneration, when the germ cells of the epithelium have disappeared, the true syncytial nature of the Sertoli tissue is very clearly shown. This tissue, even in most extreme testicular degeneration, has shown little or no indications of degeneration. It is true that many Sertoli nuclei are sloughed into the lumina, along with the degenerating germ cells, but this is due to a mechanical factor rather than to actual degenerate conditions of the nuclei themselves.

As is shown in figure 11, they do lose their normal rounded outlines and show a rather wrinkled or grooved appearance. Since no other changes have been observed, this may be due to irregular pressure from the loose fibrous and vacuolated reticulum in which they lie, rather than to degenerative conditions. This agrees with the observations of Siperstein, Allen, and Moore. Moore ('24) states that he has found 'no perceptible change' in Sertoli cells for a period of months after complete loss of germ cells. The somewhat amoeboid shapes of the Sertoli-cell nuclei have led Maximow and others to conclude that these nuclei undergo amitotic divisions. None of my observations would warrant such a statement.

Under normal conditions, the nucleus of the Sertoli cell contains a large nearly spherical body, the nucleolus (plasmosome), and close beside it a much smaller body (karyosome), termed by Allen ('19 a) the 'paranucleolus.' Thus Allen considers the nucleolus unequally bipartite in normal conditions, but in advanced stages of degeneration he finds an equally bipartiate condition, as shown in his figures 11, 13, 15, 16, and 17. I have found a small number of such equally bipartite nucleoli in practically all stages, but have not considered them a very safe criterion for degeneration. The nuclei shown in my figure 11 show clearly the normal condition of the nucleoli, although the tubule was in advanced degeneration, and probably had been so for at least several months.

The interstitial tissue

An increase in the interstitial tissue of degenerate testes has been described by Bouin ('97), Cordes ('98), Bergonie and Tribondeau ('04), Kuntz ('19), and a great many other investigators, while Siperstein ('21), Eckstein ('23), Moore ('24, '26), and others have failed to confirm this.

Allen ('19 a) finds an "increased quantity of interstitial tissue as compared with the normal" in the Osborne and Mendel rats examined, but not in the alcoholized rats of McDowell. Siperstein ('21) has not been able to demonstrate increase in interstitial tissue during either acute or chronic inanition, but in rats refed after various periods of underfeeding, he finds "a gradual increase in the relative amount of interstitial tissue until the gland regains its normal size and structure. Thereafter the interstitial tissue apparently decreases in amount until it reaches the normal proportion. That is, the interstitial cells appear relatively increased in number only during the regenerative period." Measurement of nuclei of such cells showed no appreciable difference from the normal condition.

It is true that in conditions such as shown in figure 6B the tubules are more widely separated than normally and more interstitial tissue per unit area may be seen, but this does not imply that the actual amount of interstitial tissue in the entire testis has increased. Figure 6A shows a condition similar to figure 6B, except for little or no shrinkage of the tubules, but the amount of interstitial tissue is perfectly normal in amount. The apparent increase in the case of 6B is partly due to the breaking up of the tissue into clumps, probably due to the accumulation and pressure of the watery fluid in the intertubular spaces in such testes, and partly to the decreased size of the tubules and of the testis as a whole. That is, the apparent increase is not actual, but relative.

It will be recalled that many of these testes were taken after previous removal of the other testis of the same rat. According to the well-known view of Bouin and Ancel, supported by Lipschütz, Sand, and others, if one testis is removed the other testis undergoes compensatory hypertrophy to make up for the loss, in an effort to maintain the normal amount of secretory tissue. Moore ('24) has given a review of this problem, and states that " a careful study of many different conditions of cryptorchidism by tubule measurement, histological study and weight comparisons, has failed to present any definite evidence of compensatory hypertrophy of the interstitial cells."

I have failed to observe any actual hypertrophy or hyperplasia of the interstitial tissue in testes which have developed in the absence of the other testis for various lengths of time up to 180 days, and can confirm the results of Moore in this regard.

Sexual behavior of sterile males

Throughout this work attention was given to the histological findings rather than to breeding tests of the male rats, since a great deal of latter data has been published by various workers in recent years, but practically nothing on histological study of the testes of such rats. For the past few years, Evans and Bishop at the University of California have been carrying on a study of the male sterility, with more particular emphasis on the matter of breeding tests, but have also made considerable histological studies with results similar to those obtained by the writer (personal communication).

Only eight matings of rats fed the basal ration were made in my work, so that the data are rather meager. Seven of these matings gave negative results, for the testicular degeneration was rather advanced at the time of mating. The other one did sire a litter of five young, due to the persistence of a few normal tubules in the testis which was otherwise in stage 1 of degeneration.

Observations were also made on the sexual responses of males reared on the basal ration. Females in heat were selected by the vaginal-smear method used by Evans and Bishop, and observations were made at night, on half-hour intervals of mating for each pair of animals. The tests were made from a day to three days before operation or autopsy, when one of the testes was removed for histological examination. It was found that males with testes in early stages of degeneration exhibited the normal sexual behavior, while those with advanced testicular degeneration showed little or no response, but completely ignored the females introduced into the cage.

Evans ('25) has made a more complete study, including several thousand mating tests with males after different periods of feeding on a similar basal ration or modifications of this ration. Since his results are of particular interest in this connection, I shall quote from his paper as follows:

> We have also studied the sex responses of males and analyzed the bouchon vaginale within the maximum of five minutes after its formation for the presence or absence of sperm and the detection of motility in the latter. These methods of study have enabled us to recognize broadly four stages in the development of male sterility which may be enumerated as follows: (1) Normal abundance of sperm in the bouchon vaginale; loss of fertilizing power of the sperm;—approximately normal sex responses. Toward the end of this stage abnormal spermatozoa or instances of fused spermatozoa may be found. (2) Complete loss of sperm from the bouchon vaginale; sterility. Toward the end of this period our statistics show that less than half of all matings with oestrous females lead to a 'positive' mating, whereas normally the proportion is over seventy per cent. (3) Loss of power to form the vaginal plug; there may or may not be a retention of sex responses. (4) Loss of all sex interest.

Prevention of sterility

The remarkable effect of lettuce, when added to the basal ration, in preventing the severe degenerative changes in the testis which would occur in the absence of this natural food shows the presence of some prophylactic factor in the lettuce which is necessary for the normal development and maturation of the germ cells. The question naturally arises whether this effect is due to an improved bodily condition of the animals as a result of the great increase in body weight over the normal, which these lettuce-fed rats always show, or whether it is due to the specific factor or vitamine E, which is known to be present in lettuce, and which has been shown (Evans and Bishop, '23 a; Mattill, Carman, and Clayton, '24) to be so effective in preventing and curing the sterility disorder (intra-uterine failure) in the female rat. The former authors give a good discussion (p. 278) on the existence of the E factor, and its segregation from the other known vitamines. The method of isolation of the vitamine is described by Evans and Burr ('25).

Evans ('25) has prevented male sterility by the addition of lettuce, milk-fat, beef musculature, or alcoholic extracts of such substances, to the basic ration from the day of weaning. In most of his later work Evans has used a concentrated extract from wheat germ, one drop of which is sufficient to retain fertility for about ten months and six drops sufficient to preserve fertility for an indefinite period, although litter-mate controls of such animals (on the basic ration) showed extreme testicular degeneration at the end of five months. This work shows clearly that the beneficial effect of the lettuce addition in my experiments was due to the specific factor—vitamine E.

Mattill, Carman, and Clayton ('24), who have described and pictured advanced stages of degeneration similar to my stages 4 and 5, found in testes of rats fed a milk diet high in fat, have shown that reproductive failure and testicular degeneration do not occur if the ration is supplemented with 5 per cent or 10 per cent of wheat embryo or with lettuce.

The next question is, can the addition of lettuce cure, as well as prevent, the testicular degeneration? The results of this paper show that, after degeneration has been initiated by feeding the basal ration, addition of 40 grams of lettuce daily for periods up to 150 days may prevent the degeneration of tubules not yet showing histological degeneration, but the other tubules invariably continue their retrogression to the stage of complete degeneration. None of these tubules has shown evidence of recovery, unless occasional mitotic divisions in the spermatogonia in rather degenerate tubules would be any indication of such. Mattill, Carman, and Clayton ('24) and Evans ('25) have reached similar conclusions, although the latter author finds "regeneration of the epithelium in some tubules and development of normal fertilizing power of the spermatozoa." This may be due to his longer periods of feeding the lettuce or wheat-germ-oil additions. I have observed in some of these testes, after the lettuce treatment, a number of tubules which are immature in appearance and smaller than the more normal ones; it is quite possible that prolonged lettuce feeding would eventually enable the constructive forces to gain ascendency over the destructive forces, and return the tubule to its original state of active spermatogenesis. It is of interest to note the observation of Evans ('25) that "the cure of the dietary sterility was in all instances accompanied by an improvement in the sex response."[5]

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[5] Since the preparation of this paper for publication, the recent work of Parkes and Drummond ('25) has come to my notice. These authors have described a similar condition of sterility and testicular degeneration in the albino rat, which they attribute to lack of the water-soluble vitamine B.

Their diet consists of: purified caseinogen, 20 per cent; rice starch, 55 per cent; milk-fat, 15 per cent; salt mixture, 5 per cent, and lemon juice, 5 per cent, to which was added 0.5, 1, 2, 3, 4, and 5 per cent of yeast extract (marmite). They found a decrease in the acuteness of the sterility disorder (i.e., an increase in the time required to produce definite degeneration of the testis) about in proportion to the increased amount of yeast extract added, up to the minimum amount (5 per cent) necessary for normal gonadal conditions. In other words, the smaller the amount of yeast extract supplied, the more severe are the beriberi symptoms, resulting in very subnormal growth and lowered vitality, due to malnutrition, which, in turn, expresses itself in testicular degeneration, as has been shown (p. 206) to be true in the case of inanition, deficiency diseases, and acute and wasting diseases. That is, the degeneration described by the above workers appears to be a secondary effect resulting from subnormal physiological conditions of the body as a whole, which have been brought about through insufficiency of vitamine B itself. Similarly, the testicular changes attributed by Allen ('19 a) to deficiency of vitamine B can also be more reasonably explained in the above manner.

It may be of interest to note one of the preliminary statements in the paper under discussion. After reviewing the results of Evans and Bishop on the female rat, these authors state that: "Since, however, the male rat would appear to be independent of vitamine X for effecting reproduction, the experiments to be described may be regarded as free from this complication, since they concern artificially nourished males mated to females fed on a normal diet." More

SUMMARY

1. Male rats fed the basal ration (casein 18 per cent, cornstarch 54 per cent, lard 15 per cent, milk-fat 9 per cent, salts 4 per cent, and 0.2–0.4 gram of dried yeast daily), either after weaning or after attainment of sexual maturity, maintain an excellent curve of growth usually well above that of rats fed 'natural food' diets, but reproductive failure invariably occurs.

2. The testes of such rats undergo a slow progressive desquamation and degeneration of the germinal epithelium involving chromolysis, plasmolysis, pycnosis, giant-cell formation, karyolysis, and karyorrhexis of the germ cells followed by lipoid degeneration, granular liquefaction, and final dissolution of these cells, either in the seminiferous tubules or in the ducts of the epididymis. The germ cells degenerate in exactly the inverse order of their origin. The degenerative process has been divided into five definite stages of retrogression of the germinal epithelium.

recent work on the male rat (Evans, '25; Mason, '25) shows the fallacy of such a supposition, and indicates that the male is as much dependent as the female, for normal reproductive function, upon some factor exclusive of the other known vitamines. Furthermore, the results of Evans and Burr ('25) point very strongly to the view that this new factor is of a vitamine nature, and can be segregated from all the other known vitamines. And, as yet, there is no reason to suppose that the factor required by the female is any different from that required by the male.

Their success and our failure to get normal reproduction in males on a similar diet, and with approximately the same amounts of vitamine B supplied by yeast, can be due to but two causes. Either their yeast extract, when fed at a 5 per cent level, contains sufficient of the reproductive factor for normal gonadal function or else this factor is present in some other constituent of the diet. The most probable source of this factor is the milk-fat which, in their experiments, was fed at a level of 15 per cent, as compared with 9 per cent in the case of our basal ration. It has been shown (Evans and Bishop, '23 a) that milk-fat contains a definite though low quantity of the reproductive factor, and it is quite possible that a 15 per cent level of feeding would supply sufficient for normal reproduction or at least sufficient to prevent testicular degeneration for an indefinite period, providing the general bodily conditions and growth of the rat are normal. The last-mentioned condition, then, would be regulated by the amount of vitamine B supplied and the acuteness of the beriberi symptoms resulting from this lack.

3. This testicular degeneration is similar to that described after inanition, alcoholization, chronic and wasting diseases, wound healing, deficiency diseases, experimental cryptorchidism, and x-ray treatment.

4. After completion of the process, the Sertoli syncytium alone remains. The Sertoli cytoplasm and nuclei remain normal, as far as can be determined. There is no noticeable hyperplasia or hypertrophy of the interstitial tissue. The testes are greatly atrophied, and usually turgid with a watery fluid resulting from edema and dissolution of the degenerating germ cells. The testes are often dark and hemorrhagic, due to distention of the blood vessels.

5. Different tubules of the same testis differ considerably in their susceptibility to the degenerative changes.

6. Rats from different litters vary widely in the degree of testicular degeneration attained after the same period of feeding on the basal ration. Litter mates, especially those of similar body weight, show very slight variation.

7. In rats fed the basal ration after weaning, degeneration usually begins after from fifty to sixty-five days. Sexually mature rats require from twenty-five to thirty-five days longer to initiate the process, presumably due to the greater storage of vitamine E in the tissues of such animals.

8. The duration of the process, in both cases, is approximately thirty-five to fifty days. There is no evidence that the process is either accelerated or inhibited in one testis in the absence of the other.

9. Return to a stock-food diet (dog biscuit and carrots) after degeneration has been initiated by feeding the basal ration has no beneficial effect in preventing or curing the testicular disorder—at least, not before seventy-five days of such treatment.

10. Daily addition of 40 grams of fresh lettuce to the basal ration from the beginning of feeding prevents the testicular degeneration.

11. Similar addition of lettuce to the basal ration after the initiation of degeneration cannot restore the germinal epithe-

lium to its former normal condition. But the more resistant tubules, which do not show degenerative changes at the time the lettuce asserts its beneficial effect, are preserved and may show an increase in spermatogenic activity. The other tubules continue their degeneration to the final stage. Such a condition has been termed 'partial prevention.'

12. The prophylactic effect of the lettuce, in preventing the testicular changes, is presumably due to the presence of vitamine E, and has little or no relation to the phenomenally increased growth rate of the rats. The presence of this vitamine appears to be essential for the normal reproductive function and germ-cell maturation of the male rat.

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EXPLANATION OF PLATES

Drawings for plates 1 and 2 were outlined with the aid of an Edinger's drawing and projection apparatus at an original magnification of 300 diameters. The detail was drawn in using a 2-mm. oil-immersion lens.

Drawings of plate 3 were made with the aid of a Bausch & Lomb camera lucida and a 1.9-mm. oil-immersion lens at an original magnification of 1350 diameters.

Sections were all cut 7 μ in thickness. Fixation was with Bouin and preparations were stained with iron-alum-hematoxylin and orange G except where otherwise noted.

PLATE 1

EXPLANATION OF FIGURES

1 Normal. × 130. Transverse section of seminiferous tubules of testis B 699a from a normal rat showing various phases of spermatogenesis. Note: The spaces between the interstitial tissue and the tubules are not natural. This area was selected from near the border of the section where the tubules had become somewhat separated in the process of fixation and dehydration.

2 Stage 1. × 130. Transverse section of seminiferous tubules of testis B 872a from rat on basal ration fifty days after weaning, showing first stage of degeneration. The two tubules at the left are still normal. The three at the right show the degeneration of the spermatozoa, but a normal condition of the rest of the germinal epithelium. The epithelium at X is enlarged in figure 7.

3 Stage 2. × 130. Transverse section of seminiferous tubules of testis 9222b from a normal rat of 105 days fed the basal ration for 100 days. Shows the disappearance of the spermatozoa and 'bead-like' condition of the spermatids[6] at the second stage of degeneration. The epithelium at X is enlarged in figure 8. B 15 fixation; stained with iron-alum-hematoxylin.

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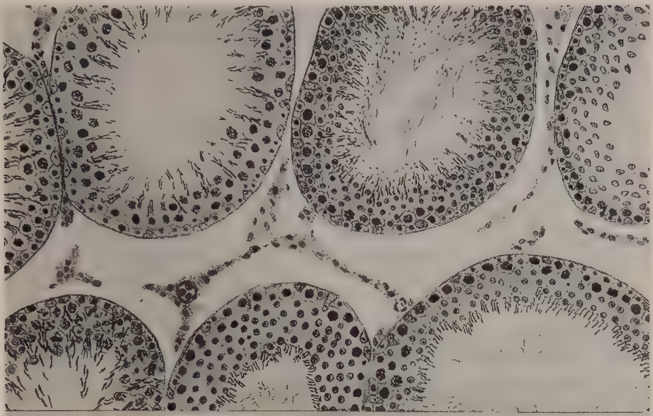
[6] See footnote on page 171.



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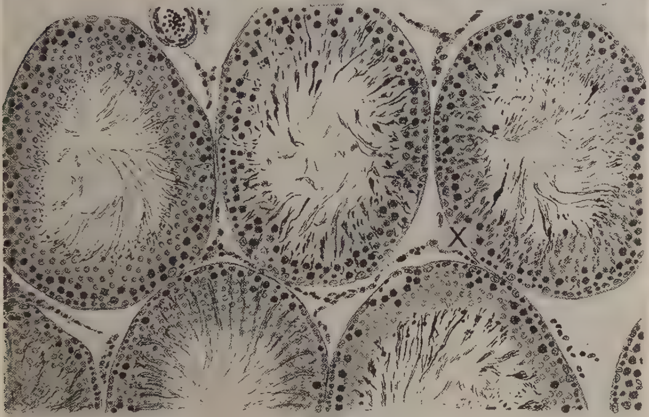

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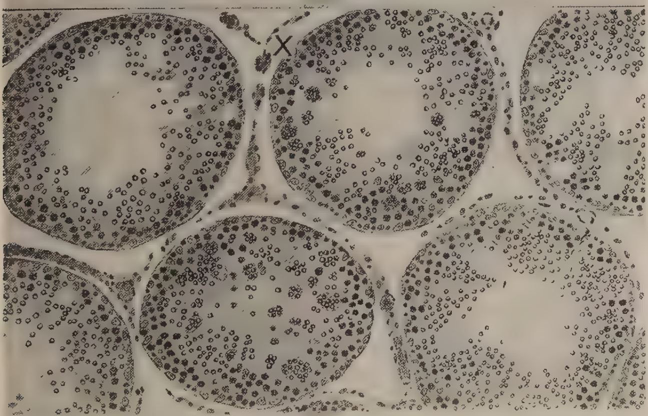

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PLATE 2

EXPLANATION OF FIGURES

4 Stage 3. × 130. Transverse section of seminiferous tubules of testis B 897a, from rat fed the basal ration for fifty days after weaning. Four tubules show the 'giant-cell' condition of the third stage of degeneration. Two of these contain masses of degenerating spermatids in the lumen. 'Giant cells' of all sizes are present in the epithelium of the tubules, one tubule (middle, lower row) shows spermatocytes in karyorrhexis, characteristic of stage 4. The epithelium at *X* is enlarged in figure 9.

5 Stage 4. × 130. Transverse sections of seminiferous tubules of testis B 696a from a rat fed the basal ration for seventy-five days after weaning. The giant cells of the previous stage have practically disappeared. The spermatocytes and spermatogonia show degenerative changes. The germinal epithelium is greatly reduced in height and the cytoplasm fibrous.

6A Stage 5. × 130. Transverse sections of seminiferous tubules of testis B 681a, from a rat fed the basal ration for eighty days after weaning. The epithelium of the tubules is reduced to a syncytium of Sertoli cells, but the size of the tubules is fairly normal. There is no hypertrophy or hyperplasia of the interstitial tissue.

6B Stage 5. × 130. Transverse sections of tubules of testis 9158b, from a normal rat of 110 days fed the basal ration for 180 days. Same condition as figure 6A, but shows much greater reduction in the size of the tubules (compare with normal condition shown in fig. 1) and clumping of interstitial tissue in irregular masses. The apparent increase in interstitial tissue is only relative to the size of the tubules. A section of the epithelium of one of these tubules is shown enlarged in figure 11. B 15 fixation; stained with iron-alum-hematoxylin and orange G.

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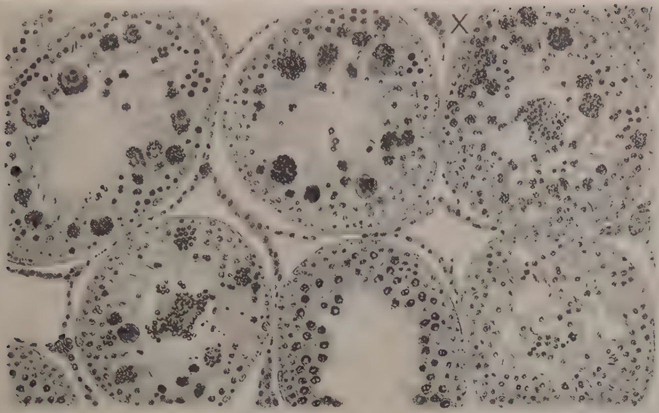

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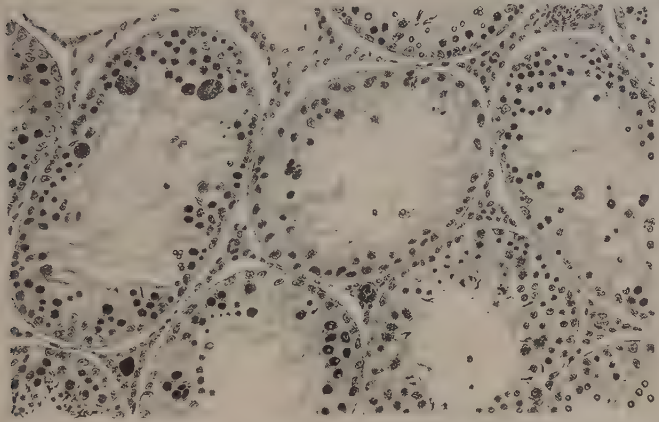

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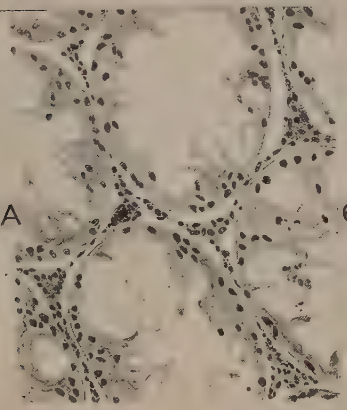

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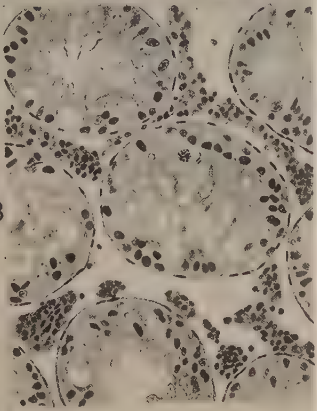


PLATE 3

EXPLANATION OF FIGURES

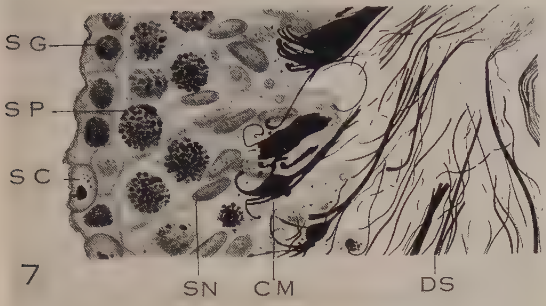
7 Stage 1. × 575. Enlarged from region *X* in figure 2. Shows detailed condition of stage 1. *SC,* Sertoli-cell nucleus; *SG,* spermatogonium; *SP,* spermatocytes; *SN,* spermatid nuclei in process of elongation preparatory to transformation into spermatozoa; *CM,* chromatin masses formed by the fusion of numbers of degenerating spermatozoa; *DS,* tails of degenerating spermatozoa.

8 Stage 2 (late). × 575. Enlarged from region *X* in figure 3. Shows absence of spermatozoa and greatly swollen condition of the germinal epithelium. *SC,* Sertoli-cell nucleus; *SG,* spermatogonium; *SP,* spermatocyte; *S,* degenerating spermatid; *B,* cytoplasmic connection between degenerating spermatids and spermatocyte; *GC,* 'giant cell' in early stages of formation, but still showing connections with surrounding cytoplasm; *SN,* spermatid nuclei in 'giant cell'; *CB,* chromatoid body of original spermatid, showing chromolysis.

9 Stage 3. × 575. Enlarged from region *X* in figure 4. *SC,* Sertoli-cell nucleus; *SP,* spermatocyte; *B,* cytoplasmic connection between two spermatids in process of fusion; *CB,* chromatoid body; *SN,* spermatid nuclei in young 'giant cell'; *GC,* 'giant cell' showing pycnotic changes; *V,* vacuoles in germinal epithelium; *DS,* degenerating spermatozoa tails.

10 Stage 4. × 575. Enlarged section of a tubule from the same slide as figure 5. Selected to show fate of large 'giant cells.' *GC,* 'giant cells' in process of lipoid degeneration and granular liquefaction; *SC,* Sertoli-cell nucleus sectioned to one side of the larger diameter; *SG,* spermatogonium undergoing liquefaction; *SP,* spermatocyte in pycnotic condition; *V,* vacuole.

11 Stage 5. × 575. Enlarged section from a tubule of figure 6B. Shows the reduction in height of the germinal epithelium and the syncytium of Sertoli-cell nuclei. *SC,* Sertoli-cell nucleus with prominent nucleolus and smaller 'paranucleolus'; *V,* small vacuoles in cytoplasm; *M,* membrana propria somewhat thickened, due to decreased size of the tubule.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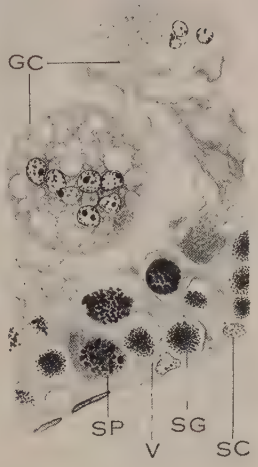
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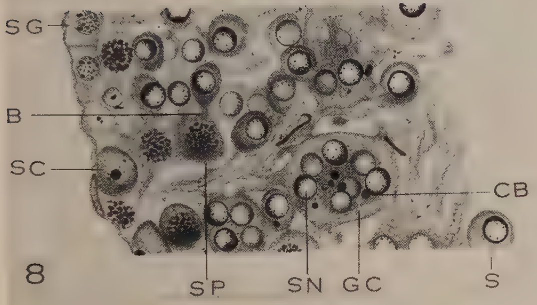
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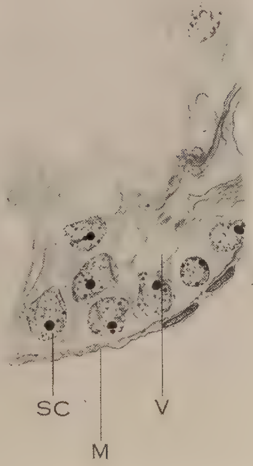
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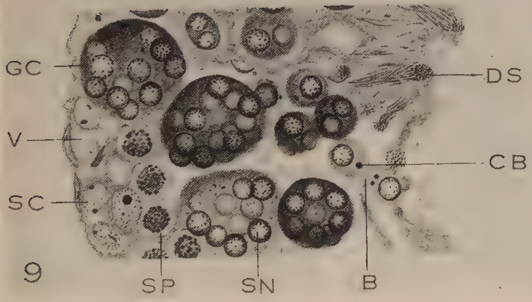
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AUTOTOMY IN ARACHNIDA

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FIVE PLATES (FIFTY-FIVE FIGURES)

AUTHOR'S ABSTRACT

On the basis of experimental study and observations on the morphology of the legs of spiders, P. Friedrich described a cutting device within the trochanter which would sever that appendage from the body when the leg was stimulated by injury.

A reinvestigation of the problem yielded entirely contrary results. Experiments calculated to induce spontaneous amputation through an automatic reflex in the leg were negative on scorpions, harvestmen, and more than a dozen species of spiders. A detailed morphological study of the skeleton and musculature of the arachnid leg showed that no autotomizing device exists, either in the skeleton or in the arrangement of muscles (as is usually supposed). In all species studied, except the scorpion, severance of the leg from the body occurs most readily at some given point. The point at which severance readily occurs is directly correlated with a definite structural weakness in the skeleton and musculature. The spider itself removes an injured leg by grasping the stump with its mouth-parts. No such injured stump falls off spontaneously or without the application of tension. Scorpions do not autotomize appendages under any stimulation, nor do they show any anatomical weakness in the leg skeleton or musculature.

Autotomy as an automatic reflex does not exist in the Arachnida.

CONTENTS

Introduction 144
Material 146
  Live material 147
  Preserved material used and methods of preparing it for study 147
Morphological study 148
  Araneae 148
    Skeleton of coxa, trochanter, and femur of Theridium tepidariorum. 149
    Musculature of coxa, trochanter, and femur in Theridium tepidariorum 151
  Opiliones 153
    Skeleton of coxa, trochanter, and femur 154
    Musculature of the coxa and trochanter 157
  Pedipalpi 158
    Skeleton of the leg 158
    Musculature of the walking leg 163
  Scorpiones 165
    Skeleton of the leg 166
    Musculature of the leg 170
Experimental study 173
  Araneae 174
    Technique 174

        Observations 174
    Scorpiones 179
        Observations 179
    Opiliones 180
        Observations 180
    Pedipalpi 180
        Observations 180
Discussion 181
Summary 184
Literature cited 185

INTRODUCTION

The first recorded observation of autotomy in the Arachnida was made by Lepeltier in 1812. Some years later, Heineken ('29), stimulated by MacCullough's work (1826) on the autotomy of claws in the crabs, made some experiments in inducing spiders to drop their legs. No explanation was offered for the phenomenon. Wagner, in 1887, made observations on the autotomy of appendages in Arachnida, and at the time recorded that the animal itself removed the injured member with its mouth-parts and remaining legs and stated that the phenomenon was a voluntary severance and not a reflex. His statements were disregarded or contradicted by subsequent writers. In watching the life-history of Phalangids, Henking ('88) remarked the ease with which the animals lost a leg when caught or injured. He ascribed this to some specially developed protective mechanism, but went no further into the subject.

Frédéricq observed in Crustacea, in 1883, and in Arachnida, in 1886, that through heat, mechanical, chemical, and electrical stimuli of the nerve, autotomy of appendages could be induced. Since all of these stimuli failed to produce autotomy when applied to the terminal segment of the leg, he concluded that the phenomenon was a reflex action controlled by the nerve itself. Knowing that the anatomical center of the leg nerve was the ventral ganglion, Frédéricq conjectured that the reaction could never be controlled voluntarily by the animal. However, it was not until 1906 that Friedrich attacked the subject from a morphological point of view. He endeavored to show that a specially developed chitinous process within the second joint (or trochanter) is the means of severing the leg. According to Friedrich, when an occasion arises for disposing of an injured member, the ventral surface of the trochanter is compressed dorsoventrally by a femoral flexor muscle, thus bringing the chitinous knife in contact first with the one intervening muscle, which it severs, and then with the interarticular membrane. The leg then is parted from the coxa, bringing off the knife with the rest of the trochanter. If the animal is not mature, a new leg regenerates in place of the lost one at the next molt. This process of autotomy, as described by Friedrich, occupied from a few seconds to many hours, depending upon a number of circumstances. Cutting of the terminal joints did not give rise to autotomy, but only when the injury was relatively near the point of severance did this phenomenon occur.

Friedrich's suggested explanation for the location of the autotomizing knife in the trochanter was that in past geologic time the spiders' legs were so often bitten off by lizards at the coxal-trochanteric joint that this device developed as a protective measure.

Owing to the fact that the literature on the musculature and the skeleton of the arachnid leg is extremely fragmentary, it was found necessary to begin the present investigation with a thorough study of the morphology of the parts involved in autotomy. Friedrich undoubtedly based his incorrect interpretation of the phenomenon on a misunderstanding of the morphology of the arachnid leg. Careful reinvestigation has proved that such as apparatus as he described does not exist, and so it became imperative to seek some other cause for the severance of the leg. The present paper is therefore divided naturally into two parts, namely, morphological and experimental. The first part deals with the normal structure of the skeletal and muscular systems of those segments in the arachnid legs which are involved in autotomy. The second part attempts not only to show on the basis of experiments the reasons for former misconceptions, but also to prove the final conclusion that autotomy in the usual sense of the word does not exist in Arachnida.

This problem was suggested by Prof. Alexander Petrunkevitch. The author wishes to express her appreciation for the interest which he has shown throughout the progress of this investigation and for his many valuable suggestions. The author wishes also to acknowledge the criticism and assistance of Mr. Horace Elmer Wood, 2nd, in the textual revision of this paper.

MATERIAL

Representatives of four orders of Arachnida permitted a broad comparative study of the group in question. With the exception of Tegenaria derhamii (T. domestica), cosmopolitan in distribution, which was the principal species used by Friedrich, all the forms listed are North American in habitat. A list of the species used follows.

- Order—Scorpiones.
  - Centrurus carolinianus.
- Order—Pedipalpi.
  - Tarantula marginemaculata.[1]
- Order—Opiliones.
  - Leiobunum negropalpi.
- Order—Araneae.
  - Family—Theridiidae.
    - Theridium tepidariorum.
    - Steotoda borealis.
  - Family—Argiopidae.
    - Araneus trifolium.[1]
  - Family—Agelenidae.
    - Agelena naevia.
    - Tegenaria derhamii.[1]
  - Family—Laterigradae.
    - Misumena aleatoria.[1]

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[1] These forms were studied from preserved material only.

Family—Pisauridae.
  Pisaurina mira.
  Dolomedes tenebrosus.
Family—Lycosidae.
  Lycosa punctulata.
  Lycosa scutulata.[1]
  Lycosa carolinensis.
  Lycosa gulosa.

Live material

With the exception of the scorpions, which were sent to the writer from Texas through the courtesy of Prof. T. S. Painter, and the Lycosa carolinensis, which were sent through the courtesy of Prof. F. Payne, of Indiana, all live material was collected in the vicinity of New Haven, Connecticut. Members of the families Pisauridae and Lycosidae were preferred for experiment on account of their larger size. The native species of harvestmen, Leiobunum negropalpi, was found frequently enough to be used for study. It was necessary, however, to get a species of spider which was not seasonal, which could readily be found in quantities large enough to allow use for both morphological and experimental study. For this purpose Theridium tepidariorum (Fam. Theridiidae) was most satisfactory. This is the common hot-house spider, which makes its web in window corners in houses, barns, and sheds. Specimens of every age are abundant and easily obtainable throughout the year.

Food for the animals was a simple matter, as they ate almost any insect offered. Musca domestica, Drosophila, and larvae of Tenebrio were easiest to procure and so were used with success. Water was given regularly to all animals.

Preserved material used and methods of preparing it for study

Specimens were prepared for the study of the skeletal parts by boiling in a strong solution of potassium hydroxide for fifteen or twenty minutes to destroy all but the chitinous parts. An entire organism was treated in this way, as it was then easier to identify the various legs. After boiling, the skeleton was rinsed in clear water and carefully dismembered with forceps and needles under a binocular. The legs were then studied either in water or in a solution of alcohol.

The best in toto mounts were prepared by fixing the animal in Bouin's fluid and separating the legs from the thorax. This fixing fluid stains all muscle tissue a deep, permanent yellow, obviating the necessity for any other stain. These legs were washed, dehydrated, and cleared in the usual manner and mounted in balsam on slides with the cover-glass supported by capillary tubes. In toto mounts made from specimens preserved in 70 per cent alcohol usually did not need a stain, but in very small delicate specimens eosin in 95 per cent proved as effective as anything. The same treatment for clearing and mounting was given these as the Bouin preparations.

Live animals were fixed in a number of fluids, Zenker's, Bouin's, Perenyi's and Petrunkevitch's, of which the last gave the most satisfactory results. It was best used while hot to insure instantaneous death, and in spite of its rapid fixing qualities it left all tissues in a perfect state of preservation. On account of the impenetrability of chitin, it was necessary to treat the organs or organisms for a much longer time than is ordinarily necessary in thoroughly exposed tissue. For staining, Delafield's haematoxylin, with eosin in 95 per cent alcohol used as a counterstain, gave satisfactory results and was used exclusively.

MORPHOLOGICAL STUDY

Araneae

The most extensive morphological study of the skeleton and musculature of the spider leg was made on Theridium tepidariorum, family Theridiidae. Additional species, given in the list above, were used for comparison and as a check against possible errors. It was found that all these spiders, which represent widely separated species and families, possess the same musculature and skeleton of the legs. However, certain distinctive minor modifications are present in each leg, which are sufficiently characteristic to permit a trained arachnologist to identify not only the leg joint itself, but also the pair of legs to which it belongs.

For the sake of uniformity, the terminology introduced by Petrunkevitch for the arachnid leg in 1909 is used here with the one simplification, suggested by him, that the terms dorsal and ventral be substituted for episynaxial and hyposynaxial.

Skeleton of coxa, trochanter, and femur of Theridium tepidariorum. Coxa. The coxa, the first joint of the arachnid leg, which has the form of a truncated cone, is slightly flattened on the pro surface and is somewhat incurved at the chitinous septum. The broader proximal end is attached to the thorax at a slightly upward-directed oblique angle. On the pro surface of the coxa in the synaxial plane dividing its anterior two-thirds into an upper and lower half is the chitinous septum, or crista (fig. 21, *C*) as it is called, which on the pro surface is seen as a heavily chitinized strip running the length of the joint and ending abruptly with it. Disjointed, however, the crista is seen to be very high at the trochanteric articulation, extending one-fifth to one-fourth the distance across the coxal-trochanteric aperture (fig. 22, *A*). The retro surface is well curved in the longitudinal plane and lacks any modification in the chitin.

Proximal articulations. The surface of attachment to the thorax on the coxa is approximately horseshoe-shaped, with two heavy articulations representing the heels (fig. 21). The crista arises on the proventral edge of the coxa in one of these articulations. The small space between these two articulations is without any heavy chitinization, but merely delineates the outer edge of the rather wide interarticular membrane which is present around the whole circumference of the coxal-thoracic aperture.

Distal articulations. The coxal-trochanteric aperture is almost heart-shaped, with the distal end of the crista representing the cleft. The only heavily chitinized portion of this attachment is the crista, the remaining articulation being but a thin interarticular membrane, the diaphragm or velum, which diminishes in width as it meets the crista. The intersegmental cleft between the coxa and trochanter is very deep, this being the point where autotomy occurs in spiders. The coxa, while tapered at its distal end, is considerably wider than the diameter of the trochanter. The thin flexible chitinous diaphragm makes up for the difference in width of the two joints and encloses the heart-shaped aperture just mentioned. This diaphragm is supported at a point diametrically opposite the crista by a sclerite, a heavy rod-like thickening in the chitin (fig. 22, *T*).

Trochanter. The trochanter, or second joint, is a very short segment. Its concave prodorsal surface is short and shows a fold in the chitin, while the retroventral surface is longer and convex. The proximal end is truncated almost squarely, but the distal, dorsal, and ventral edges curve inwardly and obliquely toward median pro and retro elevations, each of which bears an articulation. A large interarticular membrane extends from the shorter distal edge of the trochanter. When the leg is flexed, this membrane allows the trochanter to telescope over the corresponding proximal edge of the femur (fig. 32). Flexion beyond a certain limit is blocked by a fringe of stiff hairs on the trochanter at the origin of the interarticular membrane. This half of the membrane is long enough to permit extension of the leg to a full 180° and beyond. From the distal dorsal edge of this segment there also extends an interarticular membrane which, like the one just described, diminishes in width and disappears as it reaches the two articulations. This membrane is not as wide as the other, but it permits extension of the leg. In extension the proximal ventral edge of the femur telescopes over the corresponding distal edge of the trochanter.

Proximal articulations. The narrower, weaker attachment of the trochanter is at the coxa. The aperture of the trochanter, like that of the coxa, is narrower than the diameter of the segment itself. This opening is surrounded by an interarticular membrane and a chitinous rim, which is very heavy at the articulation with the crista (fig. 23, *A*).

Distal articulations. The distal aperture of the trochanter (fig. 28) is approximately oval and slightly pointed where the two articulations occur. These articulations which are opposite each other in the parasynaxial plane of the opening are the fulcra which permit the wide flexion and extension of the femur. Since there is no extensor muscle in the trochanter, extension is accomplished partly through femoral muscles and partly through the elasticity of the chitinous membranes. The articulation on the pro edge of the aperture is a shallow socket; that on the retro edge is a crude ball which fits into its complement, the socket on the femur. Excepting the points of articulation, the trochanteric-femoral aperture is surrounded by a rather wide interarticular membrane whose edge is supported by chitinized rims and whose heaviest portions are at the articulations.

Femur. The femur, or third joint, will be treated in a cursory manner in the spiders, as it is not directly connected with the problem. It is cylindrical, and usually tapers distally. The proximal or trochanteric attachment presents a rounded aperture of about the same diameter as the femur itself, having an interarticular membrane rimmed by the chitinous periphery of the femoral skeleton. Two articulations (fig. 24, A_1, A_2) are present on definitely raised points of the attachment, a ball on the pro edge to fit into the groove in the trochanter, and a groove or depression on the retro edge to receive the reciprocal ball of its articulating joint.

Musculature of coxa, trochanter, and femur in Theridium tepidariorum. There are six muscles enclosed in the coxa, five of which are entirely within the coxa, the sixth extending beyond and through the trochanter as far as the interarticular membrane of the trochanter and femur.

Muscles of coxa. The muscles belonging strictly to the coxa are:

1. Musculus levator trochanteris (fig. 1, *F*; fig. 17, *F*), a fan-shaped muscle arising partly from the chitinous septum (which is in the synaxial plane of the coxa, dividing its anterior two-thirds into an upper and lower half) and partly from the dorsal surface of the coxa and attaching itself by a tendon to the proximal dorsal edge of the trochanter.

2. M. depressor trochanteris (fig. 1, *E*; fig. 17, *E*), the antagonist of m. levator trochanteris, another fan-shaped muscle arising from the chitinous septum and from the ventral edge of the coxa and attaching itself by a tendon to the proximal ventral edge of the trochanter.

3. M. promotor trochanteris (fig. 3, *D*; fig. 18, *D*), a rather broad muscle, with parallel fibers and without a tendon at either end, arises from the ventral symmetrical surface of the coxa and attaches itself to the proximal ventral edge of the trochanter.

4. M. retractor magnus trochanteris (fig. 3, *M*; fig. 18, *M*), the antagonist of M. promotor trochanteris, another muscle without any tendon. It is broad at its proximal and narrow at its distal end, and arises from the dorsoretro proximal surface of the coxa and attaches itself to the dorsoretro edge of the trochanter.

5. Retractor parvus trochanteris (fig. 3, *P*; fig. 18, *P*), a short muscle of parallel fibers without a tendon, runs diagonally in the coxa, arising from the ventral edge of the coxa about the middle of its length, passes ventrally to M. promotor trochanteris and attaches itself to the retroproximal edge of the trochanter in a position just posterior to the attachment of m. retractor magnus trochanteris.

Muscles of trochanter. The trochanter does not provide a depressor for the femur. Depression of the femur is accomplished partly through the femoral muscle having a double tendinous origin and partly through the elasticity of the interarticular membrane between the trochanter and femur. A very weak slender muscle has no attachment in the trochanter, but passes through it. A description of the muscles contained within the trochanter follows.

1. M. levator longus femoris (fig. 1, *L;* fig. 13, *L*) arises from the proventral surface of the coxa and from the chitinous septum in the coxa, passes through the trochanter, and attaches itself to the dorsoproximal edge of the femur just retro in position to the muscle arising in the trochanter.

2. M. levator[2] bilobatus femoris (fig. 1, *B;* fig. 13, *B*), a twinned, fan-shaped muscle, with the larger portion retro in position to the smaller, arises from the ventral surface and the pro surface of the trochanter and attaches itself by a bulky tendon to the dorsal proximal edge of the femur.

Opiliones

In order to approach the study of the phenomenon of autotomy in Opiliones, it was first necessary to become familiar with the anatomy of the three proximal joints of the leg, since autotomy occurs between the second and third joints. In 1892, Paul Gaubert, in his work on the Arachnida, described the musculature of the opilionid leg as being not only homologous, but also identical with that of spiders. Investigation on the legs of the harvestman, Leiobunum negropalpi, showed that not only was the musculature unlike anything in the spiders, but also that the articulations in the leg skeleton necessitated a more primitive muscular system and less involved motion than that possessed by spiders.

A description of the skeletal parts and muscular system of the first three joints of the leg of Leiobunum negropalpi follows. It will be necessary to mention before going into the descriptions that in the case of L. negropalpi an unmodified walking leg was employed for study. All Phalangids possess on the coxa of the first pair of walking legs a masticatory process or endite. Except for minor details, however, the description applies to the coxa of every leg but the first.

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[2] M. levator bilobatus femoris is m. flexor bilobatus of the terminology of Petrunkevitch.

Skeleton of coxa, trochanter, and femur. Coxa. The coxa, the first joint of the opilionid leg, is a large heavy conical structure whose outer dorsal surface is armed with a great number of small chitinous tubercles (fig. 19). The inner side, toward the animal, is devoted almost entirely to the passage into the thorax (fig. 20). This passage is a large oval opening devoid of articulations and is defined only by a thinning of the chitin at the point of union of the segment with the thorax. The thin chitin allows the coxa to be protracted and retracted to a certain extent, but the motion is less than that of the other segments of the leg. At the distal end of the coxa at an angle of about 70° to the proximal aperture is the passage into the trochanter. As in the Araneae, the intersegmental cleft between the coxa and trochanter is much greater in relation to the diameter of the two segments than at any other joint; likewise, the passage from coxa to trochanter is much smaller in relation to the diameter of the segment bearing it. The coxa is rimmed on the ventral side by a heavy chitinous band or rebordered edge. In the synaxial plane this band sends a chitinous arm of equal thickness from both pro and retro sides upward and inward toward the aperture (fig. 19, *A*). Each arm serves as support for the articulations of the coxa. At the base of the coxa in the middorsal line a spur of unknown function is present.

The passage into the trochanter is on a rounded elevation of very flexible chitin, which is slightly higher at the ventral than at the dorsal side. This opening, which is considerably smaller than the diameter of the coxa, is oval and twice as broad as long with the longest axis in the synaxial plane. The width of the passage into the trochanter is about one-third of the diameter of the distal end of the coxa; its length is two-thirds of the diameter. During elevation and depression of the trochanter both the dorsal and the ventral edges of this aperture may be telescoped within the flexible membrane supporting it (fig. 19, *I*). The aperture is without chitinization on its circumference except in the synaxial plane at the point where the interarticular membrane is narrowest.

Here the two articulations occur, receiving support from the chitinous arms or diverticula which extend inward from the periphery of the coxa. These articulations act as fulcra for the reciprocal articulations of the trochanter and permit motion of the trochanter in one plane only, that is, elevation and depression.

Trochanter. The trochanter, or second joint, is a short truncated cone. The dorsal and ventral surfaces are slightly flattened, while the longer pro and retro surfaces curve in the longitudinal plane toward the narrower distal end. The pro and retro surfaces are armed with many chitinous tubercles resembling those on the coxa. The dorsal and ventral surfaces are of unequal length, the ventral surface being about three-quarters of the length of the dorsal surface. The distal ventral edge of the trochanter has a distinct cleft directly in the midventral line (figs. 25, 33, A_1). This cleft bears a point of articulation with the femur. At a point midway up the middorsal line a narrow thickening appears which extends toward the distal end. Inside of the trochanter this thickening is seen to be a rather high sharp chitinous septum or crista (fig. 26, *C*).

Proximal articulations. The proximal end of the trochanter presents a passageway which corresponds exactly in size and shape with the passage of the coxa articulating with it (fig. 29). The aperture is smaller than the diameter of the trochanter and so is borne on a diaphragm or velum. The passage is oval in shape and bears an oval chitinous lip on the dorsal and ventral edges of the opening. These lips are attached to the rebordered edge of the trochanter by but one side (fig. 29, *DL, VL*). The other three sides are surrounded by the interarticular membrane of the coxa and are so firmly bound to the coxa that when the leg joints are separated they come apart, adhering to the coxa as though they were a portion of it. In the synaxial plane two tubercles articulate with the reciprocal articulations described above in the corresponding plane of the coxa (fig. 29, A_1, A_2).

Distal articulations. The distal end of the trochanter presents a very restricted opening borne on a velum or diaphragm (fig. 33). This aperture is oval with its long axis in the plane of symmetry. The passage is one-third the width of the diameter of the trochanter and two-thirds its length. A wide interarticular membrane surrounds the passageway except at the median dorsal edge where it narrows and disappears. The membrane is bounded and supported at the edge of the diaphragm by a rebordered edge. This end of the trochanter bears two articulations, both in the sagittal plane. At the median ventral edge the crista presents a rather large rounded articulation (fig. 33, A_2). At the opposite dorsal edge there is a sharp rod-like articulation (fig. 33, A) which points directly toward and articulates with the cleft at the corresponding proximal edge of the femur.

Femur. The femur, the third and longest joint of the leg, is a slender, tapering segment. At the end which articulates with the trochanter, the femur is bulbous, but suddenly diminishes in size, to enlarge again gradually toward the distal end where it meets the patella, becoming about a third larger in diameter than at the proximal end. The whole shaft of the femur, except a small space at each end, is armed with tubercles. The proximal end of the femur is obliquely truncated at an angle of about 45°, the midventral surface being longer than the middorsal. This proximal articular surface forms a sharp angle at the midventral point (fig. 30).

Proximal articulations. The proximal aperture of the femur is a narrow oval, surrounded by a wide interarticular membrane. There is a cleft in the dorsal edge of the membrane with which the rod-like sclerite of the corresponding side of the trochanter articulates (fig. 30, A_1). Two small tubercles on the outer edge of the cleft reinforce the articulation. Just opposite this articulation on the ventral side, the outer edge of the membrane follows the periphery of the skeleton up to the sharp point which articulates with the reciprocal cleft of the trochanter (fig. 30, A_2).

Autotomy occurs between the trochanter and the femur, where the articulating surfaces of the two segments present very little resistance to tension. The restricted distal aperture of the trochanter facilitates blood clotting, and the muscle structure minimizes the injury done to this tissue.

Musculature of coxa and trochanter. Coxa. By the nature of the articulations between coxa and trochanter, which are in the synaxial plane, motion of the trochanter in more than two directions is impossible. The coxa contains within itself but two muscles, which are, nevertheless, very large, since they must elevate and depress the entire leg. These muscles are as follows:

1. M. levator trochanteris (fig. 10, *L*), a large fan-shaped muscle with long fibers, which arises at the ventralmost point of the coxa and along the pro and retro edges of the thoracic aperture in the coxa and attaches itself by many tendons to the lip on the dorsal proximal edge of the trochanter.

2. M. depressor trochanteris (fig. 10, *D*), the antagonist of the levator and a larger muscle of the same type. It arises just ventral to the levator muscle in the chitin along the pro and retro sides and from the ventralmost point of the coxa. The muscle attaches itself by tendons to the oval chitinous lip on the ventral proximal edge of the trochanter.

Trochanter. Two articulations similar to those of the distal extremity of the coxa are found in the distal end of the trochanter. Being diametrically opposite each other they permit motion in one plane only. The articulations on the distal end of the coxa are in the synaxial plane while those at the distal end of the trochanter are at right angles to this. The planes of movement at these two articulations are, therefore, at right angles to each other. The muscles in the trochanter, two in number, are also at right angles to those in the coxa. A description of these muscles follows.

3. M. protractor femoris (figs. 5 and 6, *P*), a large fan-shaped muscle arising on the dorsal midline of the trochanter, from the pro side of the crista, and from the entire proximal pro edge of the same segment. It attaches itself by many

small tendons to the chitinous rim of the pro half of the interarticular membrane of the proximal end of the femur.

4. M. retractor femoris (figs. 5 and 6, *R*), the antagonist of the protractor and the mirror image of it. It arises on the dorsal midline of the trochanter from the retro side of the crista, and from the entire proximal retro edge. It attaches itself by many small tendons to the chitinous rim of the retro half of the interarticular membrane of the proximal end of the femur.

No muscle from the trochanter passes beyond the interarticular membrane into the femur, nor does any femoral muscle arise in the trochanter, as is the case in the spiders and whip scorpions. Therefore, the femur contains no muscles in its proximal end. Muscles for flexing the patella have their origins further along the shaft of the femur.

Pedipalpi

In studying the leg in the pedipalp, Tarantula marginemaculata, an unmodified walking leg was used. The description given below applies to any but the first leg, which is modified into the characteristic flagellum or whip. This appendage is strictly homologous with the legs and possesses identical musculature and articulations, but differs in size and shape. It is attenuated and slender as compared with the walking legs.

For reasons which will be given later, it will be necessary to describe a greater number of segments in the leg than in the case of the spiders or harvestmen.

Skeleton of the leg. Coxa. The coxa, the first joint of the pedipalp leg, is a broad truncated cone. The ventral third of the pro side is marked longitudinally by a chitinous septum or crista (fig. 47, *C*), which arises at the base of the coxa and terminates in an articulation with the trochanter. Just below the end of the crista is a bulbous protuberance. Above the crista a constriction extends around the retro side of the segment.

Proximal articulations. The proximal end of the coxa presents a very irregular opening into the thorax which is defined along its whole periphery by a rebordered edge. There are no articulations present (fig. 46).

Distal articulations. The distal end of the coxa presents a very irregular and restricted aperture. The widest point of the passage is in the symmetrical plane of the leg; the narrowest, in the synaxial. Since the coxa is flattened in the synaxial plane, and the passage is the same shape as the end of the coxa, its width is about one-third that of the coxa (fig. 45). The aperture is borne on a diaphragm or interarticular membrane (fig. 45, *I*), which is very narrow on the pro side where the articulation occurs and very wide on the side opposite. Since there is a single articulation between these two segments, motion is possible in two planes, that is, depression and extension, protraction and retraction. The termination of the crista on the pro edge of the opening forms the articulation. A large sclerite arises on the dorsal rim of the aperture and extends diagonally across the interarticular membrane, touching the periphery of the coxa at a point slightly ventral to its plane of origin (fig. 45, *S*).

Trochanter. The trochanter, or second joint, is short, with a constricted base (figs. 47 and 48). The dorsal surface is short and concave. The convex ventral surface is about three times the length of the dorsal surface, so that the distal truncature forms an angle of 45° to the main axis of the segment. A deep concavity appears near the distal end of the ventral surface of the trochanter, which with the whole shape of the segment gives it the appearance of a coal scuttle.

Proximal articulation. The proximal end of the trochanter bears an irregular opening (fig. 39). There is no interarticular membrane at this point and the opening is bounded by a rebordered edge. A single heavy curved articulation on the proventral edge articulates with the end of the crista in the coxa (fig. 39, A_3).

Distal articulations. The distal passageway of the trochanter is a large oval opening surrounded by an interarticular membrane (fig. 40). The dorsal half of the membrane is narrow and the ventral half is perhaps twice as wide. A large curved sclerite appears in the ventral membrane in the parasynaxial plane (fig. 40, *S*). Two articulations are borne on the synaxial plane which permit motion of the femur in one plane, that is, elevation and depression. The articulation on the retro side of the aperture is a notch in the heavy chitinous rim. The opposite articulation is merely a thickened point.

Femur. The femur is the third and longest segment of the leg. It is an elongated cylinder flattened laterally, with the broadest end at the base. The whole femur is curved dorsoventrally and the dorsal surface bears a number of spines.

Proximal articulations. The proximal aperture of the femur (fig. 41) is oval, somewhat smaller than the diameter of the femur. An interarticular membrane on the dorsal and ventral edges of the opening accounts for the difference in size and also permits elevation and depression of the femur. Two articulations, one on the pro and the other on the retro side of the femur, allow this motion. The articulation of the pro side is a groove to receive the reciprocal articulation of the trochanter, while the articulation of the retro side is a point which rests in the notch of the corresponding side of the trochanter. Just below the articulation of the retro side on the rim of the skeleton is a broad projection (figs. 41 and 48, *Q*) which serves as an attachment for the femoral depressor muscle. The ventral edge of the rim has a semicircular incurvation which permits full extension of the femur. The dorsal rim is somewhat incurved from each articulation and may be telescoped within the corresponding end of the trochanter.

Distal articulations. On the distal end of the femur are two articulations. They are on the pro and retro sides of the segment a little above the synaxial plane. That on the pro side is a distinct notch which articulates with the point on the corresponding side of the patella. That on the retro side is a point which bears upon the cleft of the articulating segment (fig. 42). From the retro articulation the rim of the skeleton extends abruptly toward the base of the segment, forming a cleft. From the pro articulation a hard free flap of chitin extends partly over the interarticular membrane which bridges the gap in the skeleton left by the deep notch. The interarticular membrane allows the proximal end of the patella to telescope within the distal end of the femur when in flexion. The periphery of the femur bearing upon the patella and the edge of the flap over the membrane have a rebordered edge.

Patella. The patella, the fourth joint, is very short and of even diameter throughout its length. It is curved dorsoventrally. The sides are without modification, but the extremities show a number of irregularities.

Proximal articulations. The proximal end of the patella makes an angle of about 45° with the main axis. There are two articulations, one on the pro side, the other on the retro side, both rather near the dorsal side. The articulations correspond with those on the distal end of the femur. That on the pro side is a point (fig. 37) bearing on the notch of the femur; that on the retro side is a heavy notch receiving the reciprocal articulation of the femur. On the pro side the periphery extends abruptly downward, shortening the ventral surface of the patella. Half way between the dorsal and ventral surfaces on the pro edge there is a deep notch, a short distance away from which a sclerite arises in the interarticular membrane. This sclerite is the attachment of the patellar flexor and depressor muscles (fig. 37, *S*). The rebordered periphery of the distal end possesses an interarticular membrane on the pro side in which is the sclerite mentioned above. This membrane allows the patella to become freely flexed and telescoped within the distal end of the femur.

Distal articulations. The distal end of the patella also forms an angle of about 45° with the main axis, and is thus practically parallel to the opposite end. There are a pair of articulations, one on the pro dorsal edge, the other on the retro edge. The dorsal articulation is a thickening and the retro articulation is a point (fig. 36). The dorsal articulation is situated in a sharp incurvation which ends on the pro side in a sharp point. This point is followed by another deep incurvation which ends with the retro articulation. A small interarticular membrane is present in the incurvations. Autotomy occurs at this point and the phenomenon is to be explained partly from the fact that when stress is applied the patella and tibia come apart smoothly and with little difficulty. The two rims of the articulating joints are attached lightly and without interlacing fibers of chitin as at other intersegmental areas. Therefore slight tension serves to separate them. This locus contrasts sharply with that of the spiders where autotomy occurs between the coxa and the trochanter.

Tibia. The tibia or fifth segment of the leg is a long cylinder strongly resembling the femur, though somewhat smaller. The segment is flattened laterally and has two articulations on the proximal and two on the distal end.

Proximal articulations. The proximal end of the tibia makes an angle of about 60° to the main axis, the sharpened edge of the segment being on the dorsal surface. The opening is the same size as the diameter of the segment bearing it. There is no interarticular membrane on this end of the tibia, and motion is therefore rather restricted and in one plane. The chitin is rebordered at the periphery and bears two articulations. The largest of these is on the middorsal edge in the form of a heavy blunt point which rests in the reciprocal notch of the patella. This blunt point serves on the inner surface as an attachment for tibial extensor muscles. On the retro edge a small socket articulates with the corresponding point of the patella. A lip of thick chitin (fig. 38, *W*) is attached to the inner pro edge of the tibia and receives the attachment of the tibial flexor muscle. This lip is so lightly joined to the tibia that when tension is exerted on the tibia it separates from it and remains attached to this muscle, which arises in the patella.

Musculature of the walking leg. The musculature of the pedipalp leg as studied in Tarantula marginemaculata proved to be very similar to the musculature of the spider leg with some additional muscles. The number and location of the muscles were in many ways unlike the condition in related species of pedipalps described by Börner.

Muscles of the coxa. Three muscles for moving the trochanter arise in the coxa. A fourth elevates the femur. Except for the lack of a promotor muscle, this condition is identical with that in the spider. A description of the muscles contained in the coxa follows below.

1. M. levator trochanteris (fig. 11, *L*), a fan-shaped muscle which arises on the dorsal side of the crista and from the prodorsal proximal edge of the coxa. It is attached by a number of small tendons to the prolateral edge of the base of the trochanter.

2. M. depressor trochanteris (fig. 11, *D*), the antagonist of the levator, a broad fan-shaped muscle which arises from the ventral side of the crista and from the proventral proximal edge of the coxa. It is attached by a single tendon to the ventral proximal edge of the trochanter.

3. M. retractor trochanteris (fig. 8, *R*), a very broad fan-shaped muscle which arises from the dorsal and ventral midlines and from the proximal retro edge of the coxa. It attaches itself by many small tendons to the proximal retro edge of the trochanter.

4. M. levator longus femoris (fig. 7, *FL*), a muscle arising in two bundles, 1) along the second half of the crista and, 2) distal to the attachment of M. levator tenuis femoris on the prolateral surface of the trochanter. It is attached by a single tendon to the dorsal proximal edge of the femur.

Muscles of the trochanter. Enclosed within the trochanter are four muscles in addition to M. flexor femoris, which has one attachment arising in that segment. Three of these muscles move the femur and one moves the patella. They are as follows:

5. M. levator tenuis femoris (fig. 11, *LT*), a weak muscle of rather long fibers which arises on the prolateral surface of the trochanter and inserts itself by many small tendons into the femur along the first quarter of the length of the dorsal midline.

6. M. levator bilobatus femoris (fig. 8, *LB*), a strong, broad, fan-shaped muscle which arises on the dorsal half of the retrolateral surface of the trochanter just underneath the fibers of its antagonist. It attaches itself by two thick tendons to the retrolateral edge of the corresponding end of the femur.

7. M. depressor femoris (fig. 8, *DF*), a very strong fan-shaped muscle, the antagonist of M. levator bilobatus femoris, which arises along the ventral half of the base and also along the ventral surface of the trochanter. It is attached by many small tendons to the broad projection on the retrolateral edge of the proximal end of the femur.

8. M. flexor longus patellae (fig. 12, LP_1, LP_2), a muscle of extremely long fibers which arises in two bundles. The most proximal of these two bundles arises on the ventral half of the base of the trochanter and on the ventral surface of that segment. It attaches itself by a tendon to the ventral side of the sclerite in the prolateral interarticular membrane of the patella. The second smaller bundle arises from the distal end of the trochanter on the ventral side and, forming a long tendon, attaches itself to the sclerite along with the other bundle.

Muscle of the femur. 9. M. flexor bilobatus patellae (figs. 7 and 12, *FPB*) is a muscle made up of two bundles of fibers. One of these is composed of long fibers and arises on the retro and ventral sides of the femur and attaches itself by a tendon to the dorsal side of the sclerite belonging to the patella. The other bundle is composed of short fibers and arises on the last quarter of the ventral surface of the femur and is attached to the sclerite by the same tendon as the long fibers.

Muscle of the patella. Within the patella there is a single muscle whose fibers are spread in diverse directions. This muscle is apparently a strong one, but the lip in which its tendon is inserted is poorly attached to the tibia. When tension is exerted on the leg, the lip is separated from the tibia, causing autotomy at this point. The muscle and its attachments are as follows:

10. M. flexor tibiae (figs. 7 and 12, *FT*), a widely spread fan-shaped muscle which arises on the whole dorsal midline, along the proximal pro edge and along the ventral midline of the patella. It is attached by a thick tendon to the lip on the ventral proximal edge of the tibia.

Scorpiones

While autotomy could never be induced in the scorpion, it was necessary to become thoroughly familiar with the anatomy of its leg in order to find an explanation for this variation from other orders of Arachnida.

There is some difference of opinion as to the homology of the different joints of the leg of the scorpion with the joints of the legs of other arachnids. Kraepelin ('99), in a paper on scorpions and pedipalps, gives the following terminology to the joints; proceeding from the proximal end of the leg, coxa, trochanter, femur, tibia, and a three-jointed tarsus. Bialynicki-Birula ('17), in his work on scorpions, has a slightly different opinion of the homology and terminology and gives the following names to the joints: coxa, trochanter, femur, tibia, metatarsus, protarsus, and tarsus. The question of homology is an involved one, but, considering the type of articulation between the third and fourth joints and the muscles contained within these two segments, it was thought wise to apply Petrunkevitch's terminology, calling the fourth joint the patella and the joints following it, tibia, metatarsus, and tarsus, as in other Arachnida.

In the scorpion each pair of legs is homologous with every other pair, yet they are so graduated in size from the first to the fourth pair as to make identification of each pair easy.

The first pair of legs is about one-half the length of the fourth pair and the second and third pairs are intermediate. In addition to size differences, the first two pairs of legs may be identified by an endite on the coxa. This is a pyramidal masticatory process on the ventral surface of that joint. The endite of the first leg fits closely upon the coxa of the chelate pedipalp and the endite of the second leg fits closely over, and practically covers, the endite of the first leg.

So far as is known, no detailed description of the skeleton and articulations of the scorpion leg has been published. The description which follows below (of Centrurus carolinianus) will give an idea of a generalized leg, such as the third leg, which is an average-sized one without secondary modifications.

Skeleton of the leg. Coxa. The coxa is a thick truncated cone which is compressed laterally. The proximal end makes an oblique angle as it joins the thorax, but the distal end forms a plane which is at right angles to the main axis of the segment. The dorsal and proximal sides are one-sixth to one-third the length of the ventral and retro sides. The pro surface is marked longitudinally on its ventral third by an infolding of the chitin. This fold doubtless corresponds to the crista of the coxa of other orders of arachnids, judging by its position and relation to the muscular attachments.

Proximal articulations. The proximal end of the coxa has an irregular opening which occupies the whole diameter of the segment. It has a very wide aperture, since the passage is very oblique with relation to the main axis of the segment. There are no articulations, the joint being marked off from the thorax merely by a thinning of the chitin, and on the prodorsal edge by a short rebordered area.

Distal articulations. There are two articulations on the distal end of the coxa, bordering an oval aperture. Only one occurs at this point in the spiders and whip scorpions. Motion of the trochanter in the scorpion is confined to elevation and depression with a small amount of retraction. The crista-like infolding of the chitin on the proventral edge of the opening forms the largest articulation (fig. 34, *A*). An indentation on the retrolateral edge of the periphery forms the second and smaller articulation (fig. 35, *N*). A wide interarticular membrane surrounds the aperture, which is comparatively small with relation to the diameter of the segment. The scorpions have a deep intersegmental cleft between the coxa and trochanter as do the other three orders of arachnids studied. The diameter of the trochanter at the articulation is considerably less than that of the coxa. In elevation, depression, and retraction the dorsal, ventral, and retro edges of the trochanter telescope within the corresponding edges of the coxa. The periphery of the coxa is rebordered.

Trochanter. The trochanter is a very short thick segment with a constricted base. It is curved slightly dorsoventrally and the sides are well-rounded, but without any modifications.

Proximal articulations. The proximal end of the trochanter makes an angle of about 45° with the main axis. The trochanter, therefore, points slightly upward when the leg is at rest. The passage on the proximal end is very restricted. The opening is flattened in the synaxial plane and is without an interarticular membrane. Two articulations occur here to correspond with those in the distal end of the coxa. The larger articulation (fig. 34, *A*), that on the ventral edge, is a thickened protuberance of the rebordered periphery. The smaller articulation on the retrodorsal edge is merely a thickening on the rim of the skeleton (fig. 35, *N*).

Distal articulations. The opening on the distal end of the trochanter is at right angles to the main axis of the segment. It is oval and is surrounded by a small interarticular membrane, save where the articulations occur in the synaxial plane. This membrane makes the opening somewhat smaller than the diameter of the joint. There are two articulations in the synaxial plane. The articulation on the pro surface is a ball on the rebordered rim of the trochanter (fig. 34, *B*) which fits into a socket on the corresponding end of the femur. The articulation on the retro surface (fig. 35, *M*) is a thickening which articulates with another condyle on the corresponding end of the femur.

Femur. The femur is the longest and stoutest segment of the leg. It is slightly curved laterally and bears a number of spines or tubercles on all but the retro surface. The proximal and distal extremities are somewhat narrower than the middle of the joint. Each end bears a pair of articulations in the synaxial plane.

Proximal articulations. The pro articulation of the proximal end of the femur is a socket on the rebordered rim of the skeleton (fig. 34, *B*). The retro articulation of the same end of the femur is a heavy point which rests upon the reciprocal condyle of the trochanter (fig. 35, *M*). Surrounding the opening and between the articulations is a rather wide interarticular membrane. The central portion of the membrane is very deeply infolded, allowing sharp flexion of the femur.

Distal articulations. The distal end of the femur bears two articulations on a restricted passageway. The dorsal edge is bent downward and is heavily rebordered at this point. This prevents extension of the patella beyond an angle of 135° with the femur. The two articulations of this end of the femur are borne on prominences on the dorsoprolateral and dorsoretrolateral edges of the distal rim. Between the two prominences is an interarticular membrane, of which the ventral half is deeply infolded, thereby allowing sharp flexion of the patella. The pro articulation is a large thickening in the rebordering of the dorsolateral edge of the femur (fig. 34, *C*). It bears upon the heavily chitinized prominence in the corresponding end of the patella. The retro articulation of the same end of the femur is an extremely heavy condyle which rests in a reciprocal socket in the patella (fig. 35, *L*).

Patella. The patella shows a general resemblance to the femur. It is, however, slightly smaller. It bears a pair of articulations on either end in the same plane as those on the femur. The segment is constricted at both ends. Each extremity shows a deeply infolded interarticular membrane on the ventral side as is the case in the femur.

Proximal articulations. The dorsal half of the proximal end of the patella is heavily rebordered. At this point the heavy prominence of the dorsal side of the femur prevents full extension of the joint as indicated above. The articulations of the proximal extremity are nearer the dorsal midline than they are the synaxial plane. The articulation on the dorsoprolateral surface is a rather high prominence resting on the reciprocal prominence of the femur (fig. 34, *C*). The articulation of the dorsoretrolateral surface is a heavy socket which articulates with the reciprocal condyle of the femur (fig. 35, *L*).

Distal articulations. The pro articulation of the distal end of the patella is a small one, a ball which rests in a small socket in the corresponding end of the tibia (fig. 34, *D*). The retro articulation of the same end of the joint is a very large elevated condyle on the rebordered edge of the segment which rests in the reciprocal articulation of the tibia (fig. 35, *K*).

Tibia. The tibia is a short, slender, tapering segment. The pairs of articulations at the opposite ends of the segment are at right angles to each other. This condition is unlike that in the spiders or whip scorpions. There is a small interarticular membrane at each end between the articulations, allowing easy motion.

Proximal articulations. The articulation on the pro surface of the proximal end of the tibia is a small socket (fig. 34, *D*). That on the retro surface is a very large, heavy socket into which the reciprocal articulation of the patella fits (fig. 35, *K*).

Distal articulations. The articulations on the distal end of the tibia are in the plane of symmetry, at right angles to the articulations of the other end. The passage in the end of the tibia is a narrow oval, with a pair of articulations in the dorsoventral plane. The dorsal articulation is a thickening in the rebordering of the periphery at that point (fig. 35, *J*). The ventral articulation is a small socket on the rebordered edge in the midventral line (fig. 34, *E*).

Metatarsus. The metatarsus is a small joint very similar in size and appearance to the tibia. It is slender and tapering, with a pair of articulations on each end. The articulations of the distal end allow protraction and retraction and are at right angles to those of the proximal end.

Proximal articulations. The proximal aperture is oval and restricted, and surrounded by a narrow interarticular membrane. The dorsal articulation which bears upon the corresponding end of the tibia is a thickening in the rebordered rim of the segment (fig. 35, *J*). That on the opposite side of the opening is a small ball or condyle which rests in the corresponding articulation of the tibia (fig. 34, *E*).

Distal articulations. The distal end of the metatarsus has a very restricted oval opening. The edge is heavily rebordered and is surrounded by a narrow interarticular membrane. The articulation on the prodorsal surface is a very small thickening bearing upon a corresponding thickening in the tarsus (fig. 35, *I*). The retro articulation is an extremely heavy condyle one-third the width of the segment (fig. 34, *F*). The ventral edge of the segment has two heavy spines which prevent flexion of the tarsus beyond about 35°.

Tarsus. The tarsus, the terminal segment, is short and slender. The chitin is very thick. On the proximal end there are two articulations. The dorsal articulation is a small thickening corresponding to that in the metatarsus (fig. 35, *I*). That on the retro surface is a very large socket which articulates with the condyle of the metatarsus (fig. 34, *F*). Three claws, two large and one small, arise from a terminal ball or condyle and are moved in a socket by the muscles attached to them (fig. 34, *G;* fig. 35, *H*).

Musculature of the leg. The musculature of the leg of the scorpion, although bearing some resemblances to that of the spider and whip scorpion, shows some marked differences. It is reduced when compared with either, though some few muscles are undoubtedly homologous with those of the other two orders. In 1892, Gaubert studied the muscular system of the scorpion leg and described it as being identical with that in several orders of Arachnida. The present study shows that this is not the case with the common scorpion, Centrurus carolinianus, and it is very doubtful whether other species would show such close similarity to otherwise distantly related orders of arachnids.

For reasons which will be explained later, autotomy does not occur in the scorpion. In view of the fact that no special group of joints is involved in this part of the investigation and since a complete description of the musculature of the scorpion leg is lacking, the muscular system of the whole leg is presented in the next few paragraphs.

Muscles of the coxa. Enclosed within the coxa are three muscles. Though they do not arise in exactly the same manner, they have the same attachments and functions as the three muscles in the coxa of the pedipalp leg. In spiders one or two additional muscles are present which move the trochanter; in the harvestmen there are but two muscles in this segment, a levator and a depressor. The muscles of the scorpion leg are as follows:

1. M. levator trochanteris (figs. 2 and 4, *L*), a fan-shaped muscle which arises on the dorsal and retrodorsal surfaces of the coxa and attaches itself by a tendon to the dorsal edge of the base of the trochanter.

2. M. depressor trochanteris (figs. 2 and 4, *D*), a fan-shaped muscle which arises on the ventral side of the crista, along the proventral edge of the base and also the ventral surface of the coxa. It attaches itself by a tendon to the ventral edge of the proximal end of the trochanter.

3. M. retractor trochanteris (figs. 2 and 4, *R*), a small fan-shaped muscle which arises on the retro edge of the base of the coxa and attaches itself by a few small tendons to the retro edge of the proximal end of the trochanter.

Muscles of the trochanter. Two muscles arise in the trochanter which move the femur. A few fibers of a third muscle which moves the patella also have their origin in this segment. A description of these muscles follows.

4. M. flexor longus femoris (figs. 2 and 4, *FL*), a fan-shaped muscle which arises in two bundles. The longer bundle of fibers arises in the region of the crista of the coxa on the proventral surface; the shorter but thicker bundle of fibers

arises on the pro surface of the trochanter. Both of these attach themselves by a common tendon to the dorsal edge of the proximal end of the femur.

5. M. depressor femoris (figs. 2 and 4, *DF*), a weak fan-shaped muscle which arises on the retrodorsal surface of the trochanter and attaches itself by a tendon to the ventral edge of the base of the femur.

Muscle of the femur. There is but one muscle entirely enclosed within the femur. This is less than the corresponding musculature in the spider or whip scorpion. The patella lacks an extensor.

6. M. flexor bilobatus patellae (figs. 2 and 4, *FPB*), a muscle whose fibers arise in two bundles. The smaller but longer bundle arises on the retroventral surface of the end of the trochanter and joins the other bundle of fibers which arises along the dorsal surface of the femur. These attach themselves by a common tendon to the ventral proximal edge of the patella.

Muscles of the patella. Three muscles have their origin in the patella. Two of these move the tibia and one the tarsus. Only one of these, the flexor, might be compared to a muscle of similar function in the spiders and whip scorpions. The flexor of the tarsus arises in the patella in this order, one segment proximal to the origin of the same muscle in the two orders just mentioned.

7. M. flexor tibiae (figs. 2 and 4, *FT*), a muscle of widely spread fibers, some of which arise in the distal end of the femur and some of which arise on the ventral and prolateral surface of the patella. This muscle attaches itself by a single large tendon to the ventral edge of the tibia.

8. M. extensor tibiae (figs. 2 and 4, *E*), a long muscle which arises from the dorsal distal end of the patella and from the retrolateral surface of the same segment. It is attached by a tendon to the dorsal edge of the proximal end of the tibia.

9. M. depressor tarsi (figs. 2 and 4, *DT*), a long slender muscle depressing the terminal joint and the metatarsus as

it passes through it. It arises on the retrolateral surface of the distal end of the tibia and, soon forming a long thick tendon, attaches itself to the base of the two claws in the tarsus. Three muscles arise in the tibia, two of which move the metatarsus. The third extends the tarsus. They are as follows:

10. M. promotor metatarsi (fig. 2, *PM*), a fan-shaped muscle which arises on the ventral and prolateral surfaces of the tibia and attaches itself by tendons to the prolateral edge of the base of the metatarsus.

11. M. retractor metatarsi (fig. 4, *RM*), a fan-shaped muscle which arises on the dorsal, ventral, and retrolateral surfaces of the tibia and attaches itself by tendons to the prolateral edge of the base of the metatarsus.

12. M. extensor tarsi (figs. 2 and 4, *LT*), a very small, slender elongate muscle present both in the spiders and whip scorpions. It arises on the prolateral and ventral surface of the distal end of the patella, passing through the metatarsus, and attaches itself by a very long tendon to the base of the claws in the tarsus.

EXPERIMENTAL STUDY

As was pointed out in the introduction to this paper, any careful examination of the spider leg shows convincingly that such a device as Friedrich described for amputating the leg does not exist. Since the study of the skeleton and musculature of the leg gave no hint of any specific or specially adapted cutting apparatus, the next step was to analyze by experiment other means of inducing autotomy. In full expectation of positive results, diverse experiments were performed. A careful record was kept as to the conditions and reactions, and later the results were correlated with the morphology of the leg. Partly on the basis of previous work, a number of possibilities were formulated upon which experiments were based to find out whether spontaneous amputation of the leg was due to any of the following causes: 1) a cutting apparatus of any kind; 2) constriction of the interarticular membrane by muscular contraction which would act as a kind of sphincter, which would also aid in stopping the blood flow; 3) muscular activity, such as torsion or antagonistic action in such a way as to separate the joints; 4) loosening of muscle attachments and of the interarticular membrane through enzymatic action of the injured leg.

Araneae

In the experimental work on live spiders, only native species were used. Several widely separated families were represented, so that the conclusions drawn from the results can be applied fairly to the whole order of Araneae. All recorded observations agree that autotomy in spiders always occurs between the first and second joints, that is, between the coxa and trochanter. This was the case in all species examined.

TABLE 1

Severance of the leg when grasped with forceps

SPECIES	NUMBER OF ANIMALS	TIME REQUIRED	WHERE GRASPED
Agelena naevia	8	10 to 60 seconds	Grasped by metatarsus or tarsus
Lycosa carolinensis......	8	Instantaneous	Grasped in middle of femur
Lycosa gulosa...........	5	Instantaneous	Grasped in middle of femur
Theridium tepidariorum	47	15 to 90 seconds	Grasped by metatarsus or tarsus
Theridium tepidariorum	21	Instantaneous	Quick grasp of femur
Pisaurina mira..........	5	Instantaneous	Quick grasp of femur
Steotoda borealis........	3	Instantaneous	Quick grasp of femur

Technique. The experiments, though virtually conclusive, were simple. They consisted in: 1) pulling or holding the leg until it was released by the spider; 2) mutilating it by crushing or cutting to produce autotomy through injury; 3) electric stimulation to produce contraction of specific muscles; 4) mutilation of such a character as to prevent automatic amputation and autophagy.

Observations. The experimental results will be expressed in tabulated form below. So far as possible, each animal received but one injury. When material was limited, such as the large Lycosae, it was necessary to use the same animal for more than one experiment. In this case the injuries were made several weeks apart to allow sufficient time for recovery. As a rule, the mortality was low, and when it occurred it was due largely to other causes than the loss of a leg.

These observations are analyzed further on. As the animal struggled, a break was felt within the leg before the chitinous parts separated. Histological material, preserved before complete separation had occurred, showed the release of the musculus flexor longus femoris from its attachment. The broken fibrils were visible within the sarcolemma which was not severed (fig. 51, *Z*).

TABLE 2

Autotomy induced through injury

SPECIES	NUMBER OF ANIMALS	TYPE OF INJURY	AUTOTOMY
Agelena naevia	3	Leg cut at tibia	Pulled off by animal
Lycosa carolinensis	1	Leg cut at tibia	Pulled off in 1 hour by animal
Lycosa punctulata	2	Leg cut at tibia	Pulled off in 1 hour by animal
Lycosa carolinensis	3	Leg cut at metatarsus	None
Lycosa carolinensis	3	Leg cut at tarsus	None
Pisaurina mira	4	Leg cut at metatarsus	None
Pisaurina mira	1	Leg cut at tarsus	None
Pisaurina mira	1	Leg cut at femur	Animal struggled to get member in mouth, finally constructed web such as is made in molting, and pulled off leg
Theridium tepidariorum	25	Leg cut at femur	Pulled off by animal
Theridium tepidariorum	3	Leg cut at tibia	Pulled off by animal
Theridium tepidariorum	2	Leg cut at metatarsus	None
Theridium tepidariorum	32	Leg cut at tarsus	None

Extreme muscular exertion occurred in the animal while it was being held in this experiment. It was not clear whether the animal was merely struggling to escape or whether it was perhaps assisting an inner torsion or antagonistic action of the leg muscles.

In this series of experiments the injury was instantaneous and the animals' behavior was independent of any outside stimulus other than the injury. When the tarsal joints were injured, the spider invariably thrust the injured member into its mouth and licked off the drops of blood that appeared. Injury to the two terminal joints resulted in no further reaction on the part of the organism. Amputation at the tibia and proximal to that point invariably resulted in the loss of that leg. Careful watching revealed the fact that no animal dropped its leg without aiding the procedure with its other legs and with its mouth-parts. Many individuals struggled intermittently for several hours, pushing the injured stump toward the mouth. When the efforts succeeded, the leg was severed from the body, and in the greater number of cases was digested and eaten. In one instance a Pisaurina mira, after repeated struggles to amputate the leg stump, constructed a sheet-like web such as is woven at the time of molting. It then climbed on the web in an inverted position and by manoeuvering for a time succeeded in separating the limb from its body. The limb was then eaten with the same alacrity as though it had been an ensnared fly.

Though these results were highly suggestive, nevertheless they were not conclusive as to whether any anatomical device for amputating the leg existed, and therefore experiments with electric stimulation were employed in order to decide whether real autotomy could ever be induced by an antagonistic action of the muscles. A muscle exists in the spider leg (m. flexor longus patellae, homologous with the muscle of the same name in the whip scorpion) which has two origins. It arises on the ventral surface of the distal end of the trochanter and from the ventral proximal end of the femur, and functions to flex the patella. It was conceivable that the double origin of this muscle in the trochanter could so antagonize the action of the trochanteric muscles as to sever the leg at the interarticular membrane of the coxa and trochanter. By the use of an induction coil and fine electrodes, the area of the femur containing the muscle in question was stimulated with varying strength of current. The legs of four large Lycosae carolinensis and of two large Dolomedes tenebrosus were stimulated with negative results. When a very weak electrical stimulus was applied, no response occurred. A stronger current partially disabled the leg, and a very strong current paralyzed the leg completely. The animals submitted to this treatment recovered partial use of the leg in a day or so, but died within a few days. Coxal and trochanteric muscles were stimulated in the same manner with the same negative results.

Having observed that the spider, when left to itself after a mutilation of the leg, would amputate the stump with the help of its remaining legs and of its mouth-parts, and that no true autotomy could be induced with the aid of electrical stimulation, two types of experiments were performed to decide the question definitely. The first of these was to exert pressure, but not tension, upon the femur. This was accomplished by holding the spider firmly upon a table, supporting the coxae of one side of the animal upon a glass slide and pressing the femur against the slide with a blunt instrument. In the spiders, as in other groups of Arachnida, the femur is the stoutest and largest of the leg joints. It is thick as well as long. When the heavy femoral skeleton was compressed, the indentation caused a shortening of the cylinder in a longitudinal direction and a spreading and flattening of the basal and distal periphery. A paper cylinder when crushed in the middle will illustrate this result. As the trochanter is a very short rigid joint, firmly attached to the base of the femur, it participates in the distortion of the femur. Likewise, the coxa is a short rigid joint; but its attachment to the trochanter, as compared with other joints, is anatomically weak and this articulation does not remain intact under the strain exerted by the change in the shape of the trochanter when an adjoining part of the femur is compressed.

The anatomical basis for the break at this point will be discussed later in detail. Suffice it to say here that it is natural to assume, on the basis of morphology, that the coxal-trochanteric articulation is the point at which a break would occur. This explains the instantaneous severance of the leg when the femur is suddenly grasped.

A second operation was performed calculated to prevent self-mutilation or autophagy after injury, but to allow automatic amputation to take place. This consisted in cutting off the leg at the base of the femur so as to prevent the stump from being reached by the chelicera or legs, but to allow coxal and trochanteric muscular activity. Fifteen spiders of varied species were cut in this manner, and not one of them lost the remaining segments. The reaction of the injured animal was to dip the pedipalps into the bleeding stump and lick the fluid off. This it repeated indefinitely. Several animals so injured died from loss of blood and infection due to inability to form a clot over so large a bleeding surface.

Since the articulation between the coxa and trochanter is structurally different and weaker than any other intersegmental area, a device for measuring the relative strength of the joints was constructed. A spider, Lycosa carolinensis, whose cephalothorax measured 12 cm., was etherized and put upon a tin plate with a hole through which one leg was passed. To the leg were tied three threads from which a small metal cup was suspended. The cup was filled with shot until the leg was parted from the body of the spider. The severed leg was then suspended by its tarsus and a larger aluminum cup attached to the trochanter by threads. This cup was completely filled with shot before any of the segments separated. The joint which first separated was the metatarsal-tarsal. The weight required to dismember the leg from the body at the coxal-trochanteric joint was 14 grams. That required to separate the next weaker joint was 220 grams. In terms of percentage, the coxal-trochanteric joint is less than 7 per cent as strong as the next weaker joint, the metatarsal-tarsal. Proportionate results were obtained with smaller individuals.

Mention was made by Friedrich and others of a chitinous plug or stopper which formed at the stump of the injured coxa to prevent bleeding. It was known from observation that a heavy blood clot formed at the aperture, but the question of the presence of an actual chitinous structure arose. In animals bearing an old scar, this appeared to be a chitinous plug varying in color with age. In serial sections (fig. 49, *P*) this structure took no stain and bore the yellow structureless appearance of old chitin. Upon boiling old autotomized coxae of Epeira trifolium and of a tarantula in KOH (which destroys all tissues but chitin) the unmodified coxa remained in each case without any chitinous plug. The mass over the aperture of the stump, therefore, should be interpreted as a blood clot which becomes oxidized and heavily pigmented upon exposure to air.

Scorpiones

Observations. Practically the same types of experiments were carried out on the scorpion as on the spider. Fifteen animals in all were experimented upon, each being used for more than one experiment. Holding, crushing, or cutting the leg had no effect in inducing autotomy or in stimulating the animal to chew or pull off its leg. The types of injuries are tabulated below.

TABLE 3

Centrurus carolinianus

NUMBER OF ANIMALS	INJURY	REACTION
6	Animal suspended by femur with forceps	Break occurs at point where held, in middle of joint, or at trochanteric-femoral articulation.
8	Animal suspended by metatarsus with forceps	Leg twisted off at tibial-metatarsal joint
3	Animal suspended by patella with forceps	Leg twisted off at patellar-femoral joint
2	Leg cut at trochanter	Animal died in one week
3	Leg cut at coxa	Animal died in one week
3	Leg cut at femur	No reaction
4	Leg cut at patella	No reaction
4	Femur crushed with forceps	No reaction; leg continued in use, but stiff beyond femur

Neither mutilating nor pulling the scorpion leg resulted in its severance at a definite joint as in the spiders. Drastic pulling had to be resorted to before the leg would part from the body. The break occurred at the joint just proximal to the point where the forceps were applied. In some cases the leg broke in the middle of the segment. These results can be traced directly to the anatomical structure of the leg. In the scorpion the chitinous structure at all the joints of the leg is equally strong, and the muscular strength tends to be more evenly distributed than in the joints of other arachnids.

Opiliones

Observations. Because of shortage of live material in winter, investigation on the Opilionids was largely confined to anatomical study. Some dozen animals showed that even the slightest tension on the leg caused it to part from the body, always at a definite point, the trochanteric-femoral articulation. The anatomical study showed that there was no special apparatus for cutting the leg at that point, nor was there an arrangement of the muscles which could be used in an antagonistic or torsive action. It will be explained later how the weakness of the leg at this point is dependent upon the skeletal and muscular structure. The attachment of the femur to the trochanter bears a certain similarity to the attachment of the trochanter to the coxa in the spiders.

Pedipalpi

Observations. The study of autotomy in the Pedipalpi was confined entirely to an investigation of the morphology of the leg. For this work preserved animals which had autotomized legs were obtained through the courtesy of Professor Petrunkevitch. The skeleton and muscular system of both normal and dismembered legs were carefully examined, and in the light of the earlier experimental work and morphological study safe conclusions can be drawn. Certain similarities are present between the pedipalp leg and the spider leg, but also certain differences prevail which prevent the breaking of the leg at the coxal-trochanteric joint, but which favor a break much farther down the leg, between the patella and tibia. This break occurs, as in the spiders and harvestmen, because of a weakness in skeletal and muscular structure. It is not known whether or not the pedipalp aids the amputation with its other legs. The spider possesses a well-defined instinct to tug at the injured member until it comes off. The scorpion lacks any point where severance of the leg occurs naturally and also the instinct to pull at an injured leg.

DISCUSSION

Since it has been demonstrated experimentally that autotomy in certain Arachnida is not an automatic reflex, accomplished either by a specially provided apparatus or by muscular contraction, it remains to connect the results of the experiments with the morphological observations on the joints at which the amputation occurs. There are certain marked similarities in the articulation of the joints which are involved in the severance. These similarities in articulations hold for the three orders of Arachnida observed which lose the legs at a specific point.

In studying the skeleton of the spider leg, it was observed that separation of the trochanter from the coxa was accomplished with ease. This is unlike other intersegmental points in the leg. The break at the end of each segment is clean and sharp, with only a few frayed fibers of chitin which interlace at the joint. The surfaces of articulation on the coxa and trochanter are lightly joined and slightly rebordered (fig. 50, *W*). The situation is different at other joints, where separation is relatively difficult and where numerous interlacing fibers of chitin prevent an easy, clean break. The point where severance occurs presents the smallest possible surface of injury. The intersegmental cleft is deep and the aperture is small, so that when injury does occur the small opening allows easy clotting of the blood and prevents any great loss of the fluid. Sections made of partly disjointed legs showed the tearing of the insertion and body of one intervening muscle between the coxa and trochanter (fig. 51, *Z*). This muscle, m. flexor longus femoris, remains with the coxa. The other muscles contained in the coxa which were attached to the base of the trochanter, remained intact in the coxa, with their insertions removed from the trochanter, but uninjured. Certainly, a minimal amount of injury is done to the remaining muscles.

Not so much because of stronger muscle attachments, as because of uniformity in the chitinous structure at the joints, autotomy could not be induced in the scorpion. No specific joint is more susceptible to separation than any other. In dismembering the leg skeleton at the joints, it was extremely difficult to separate each segment intact without a part of the adjoining segment adhering. The intersegmental areas are firmly bound together by chitinous fibers, and when the joints are torn apart the edge of the aperture bears frayed fibers which have come from the next segment. This interlacing of the structure is so uniform throughout the leg that no weakness occurs in the articulation; therefore, there is no point susceptible to accidental amputation.

In the phalangids there is a similarity in the structure of the joint between the trochanter and femur to the joint between the coxa and trochanter in the spiders. Here the joints, as in the Araneae, are but loosely united, without any interlacing fibers of chitin passing from one segment to the other (fig. 54, *W*). In other joints of the same leg it is only with difficulty that the segments can be separated intact. The femur is relatively rigid in its attachment to the trochanter. Such flexion as is possible between the two joints must be accomplished through the interarticular membrane of the trochanter (figs. 53, 55). However, the two joints are joined very lightly (fig. 54, *W*) and slight tension exerted anywhere on the leg will sever it at the trochanteric-femoral articulation.

Severance of the opilionid leg is accomplished without injury to a single muscle. When amputation of the leg occurs, the insertions of the two muscles protracting and retracting the femur remain in the trochanter. As no muscle passes through the trochanteric-femoral passage, no further injury is done to the remaining tissues. The aperture exposed after amputation is very small and encourages clotting of the blood by its narrowness.

A possible explanation of the location of this weak joint as compared with the spiders is that in the evolution from the crustaceans a different pair of segments fused to form the trochanter than in the spider. A crista is present in the trochanter of the harvestmen which is a point of attachment for protractor and retractor muscles. The crista in the coxa of the spiders acts as an attachment for the muscles of the same function. The levator and depressor muscles are in the coxa in harvestmen. Muscles which correspond to these four are contained within the coxa of the spider.

While the morphology of the leg joints of the pedipalp, Tarantula marginemaculata, is in some ways unlike that of other arachnids, nevertheless the point at which severance occurs is similar in structure to the same area in the spiders and harvestmen. The disjoining of the leg occurs far down, between the patella and tibia. This point lacks strength of articulation and interlacing fibers of chitin, and motion is possible in one direction only, that is, flexion. When the break occurs, both articulating surfaces of the separated segments come apart clean. The interarticular membrane here is very narrow, making the joint more or less inflexible. One muscle is disturbed, but not injured in autotomy. When the leg is torn apart, the single muscle which arises in the patella brings its insertion with it (figs. 7 and 12, *FT*) and remains whole within the patella. No other muscle is disturbed since the nearest muscle arises farther down the tibia. All the legs which were found 'autotomized' show this condition. It is quite clear that no specially adapted apparatus exists for cutting off the leg of this pedipalp. Likewise, the one muscle present in the patella could not separate the leg. Autotomy must therefore be due to accidental tension or to autophagy when the leg is injured. As compared with the spiders, the muscles of this joint are greatly reduced. In addition to the flexor, there is a depressor and a metatarsal flexor. This reduction in muscles would also partly explain the weakness of that joint.

It has been demonstrated morphologically that the weakness in articulation of the joints where autotomy occurs is directly responsible for the locus of the break. This severance is primarily dependent upon the chitinous structure of the leg rather than on the muscular system, although the latter has some bearing upon it. In each case the break occurs where the muscles will be least injured. The question of tearing other soft organs, such as blood vessels and nerves, is a minor one. Each of these would require very little tension to sever it. Their position and relation were not investigated, being considered irrelevant to the problem.

The experimental investigation points conclusively to the fact that autotomy is never automatic, but is in reality the result of tension which the structure cannot withstand. It may also be an amputation performed by the spider when the leg is injured. A relatively severe injury is necessary to stir the animal to pull off the remaining stump. Injuries to the tarsus and metatarsus, and sometimes to the tibia, were borne by the spider without promoting autophagy. In fact, it is not unusual to find wild spiders with legs thus injured, but not shed. It is easy to interpret the ability to sever their own legs as a protective measure. At the same time the conclusion seems to be unavoidable that true automatic severance of limbs in the Arachnida does not exist.

SUMMARY

1. The morphology of the legs was studied in four orders of Arachnida.

2. Neither the apparatus described by Friedrich, nor any other apparatus specially adapted for autotomizing the leg, exists.

3. In spiders there is a direct correlation between the structural weakness of the coxal-trochanteric articulation and the fact that the leg separates from the animal at this point.

4. In the harvestmen there is a direct correlation between the structural weakness of the trochanteric-femoral articula-

tion and the fact that the leg separates from the animal at this point.

5. In Pedipalpi there is a direct correlation between the structural weakness of the patellar-tibial articulation and the fact that the leg separates from the animal at this point.

6. In scorpions the equality of strength in the skeletal and muscular structure of the various joints of the leg is correlated with the absence of any specific locus of severance.

7. Reflex muscular activity is not the cause of autotomy.

8. So-called autotomy is the inability of a leg to withstand more than a certain degree of tension. It may include self-amputation or autophagy, in cases of severe injury to the leg.

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PLATE 1

EXPLANATION OF FIGURES

1 Left leg of Theridium tepidariorum, pro surface.
3 Right leg of Theridium tepidariorum, retro surface.

B, m. flexor bilobatus femoris
D, m. depressor trochanteris
E, m. extensor trochanteris
ET, m. extensor tarsi
F, m. flexor trochanteris
FB, m. flexor bilobatus patellae
FL, m. flexor patellae
FM, m. flexor metatarsi
FR, m. flexor tibiae
FT, m. flexor tarsi
L, m. flexor longus femoris
M, m. retractor magnus trochanteris
P, m. protractor tibiae
PP, m. retractor parvus trochanteris
X, point at which autotomy occurs

2 Third right leg of Centrurus carolinianus, pro surface.
4 Third right leg of Centrurus carolinianus, retro surface.

D, m. depressor trochanteris
DF, m. depressor femoris
DT, m. depressor tarsi
E, m. extensor tibiae
FL, m. flexor longus femoris
FPB, m. flexor bilobatus patellae
FT, m. flexor tibiae
L, m. levator trochanteris
LT, m. levator tarsi
PM, m. promotor metatarsi
R, m. retractor trochanteris
RM, m. retractor metatarsis

5 Muscles arising in the trochanter of Leiobunum negropalpi, dorsal view.

A, dorsal articulation of the coxa with the trochanter
C, crista
P, m. protractor femoris
R, m. retractor femoris

6 Muscles arising in the trochanter of Leiobunum negropalpi, ventral view.

A, ventral articulation of coxa with trochanter
P, m. protractor femoris
L, ventral lip of trochanter, attachment for trochanteric depressor muscle
R, m. retractor femoris

7 Muscles in pro surface of leg of Tarantula marginemaculata.

FL, m. flexor longus femoris.
FPB, m. flexor bilobatus patellae
FT, m. flexor tibiae

8 Muscles arising on retro surface of coxa and trochanter of Tarantula marginemaculata.

DF, m. depressor femoris
LB, m. levator bilobatus femoris
R, m. retractor trochanteris

9 Distal end of the coxa of Leiobunum negropalpi.

A_1, pro articulation of coxa with trochanter
A_2, retro articulation of coxa with trochanter
I, interarticular membrane

10 Muscles arising in pro surface of coxa and trochanter of Leiobunum negropalpi.

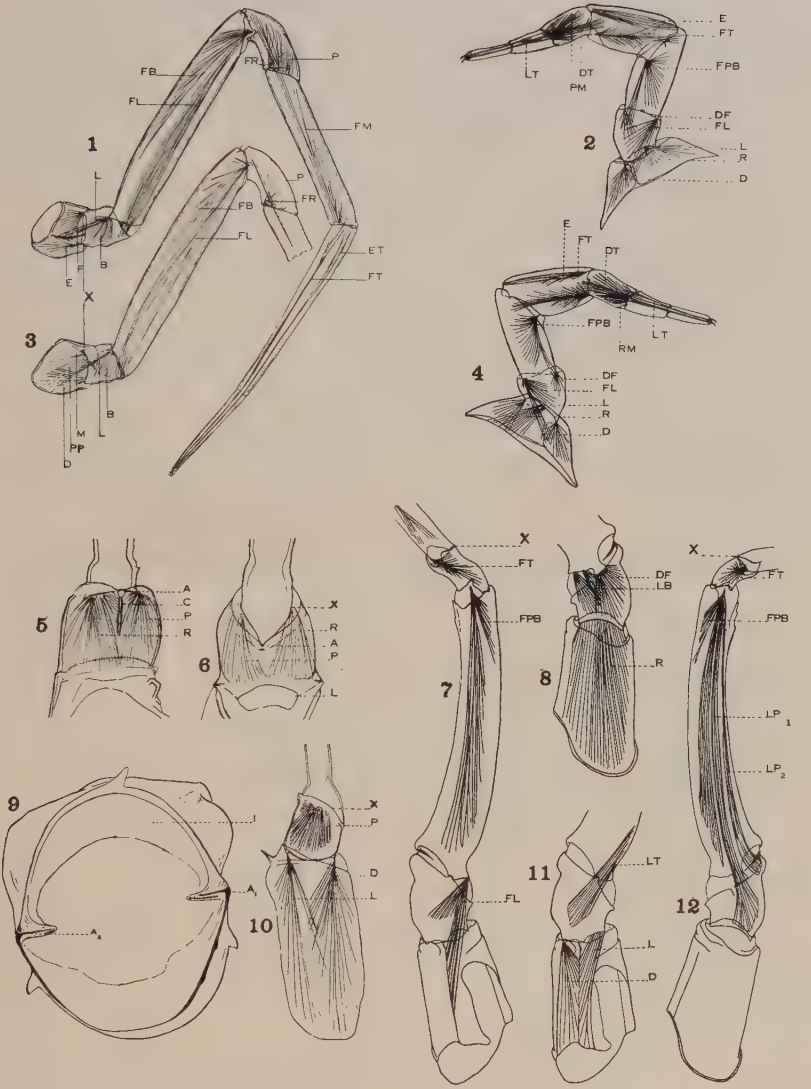
D, m. depressor trochanteris
L, m. levator trochanteris
P, m. protractor femoris

11 Muscles arising in pro surface of coxa and trochanter of Tarantula marginemaculata.

D, m. depressor trochanteris
L, m. levator trochanteris
LT, m. levator tenuis femoris

12 Muscles arising in retro surface of leg, Tarantula marginemaculata.

FBP, m. flexor bilobatus patellae
FT, m. flexor tibiae
LP_1, LP_2, m. flexor longus patellae
X, point at which autotomy occurs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LP_1
LP_2

PLATE 2

EXPLANATION OF FIGURES

13 Muscles arising in pro surface of coxa and trochanter, Theridium tepidariorum.
14 Same from retro side.

B, m. flexor bilobatus femoris. *L*, m. flexor longus femoris.

15 First three joints of leg, pro surface, Leiobunum negropalpi.

A_1, dorsal articulation of trochanter with femur
A_2, pro articulation of coxa with trochanter

16 First three joints of leg, retro surface, Leiobunum negropalpi.

A_1, ventral articulation of trochanter with femur
A_2, retro articulation of coxa with trochanter

17 Muscles arising on pro surface of coxa of T. tepidariorum.
18 Muscles arising on retro surface of coxa of T. tepidariorum.

D, m. depressor trochanteris
E, m. extensor trochanteris
F, m. flexor trochanteris
L, m. levator longus femoris
M, m. retractor magnus trochanteris
P, m. retractor parvus trochanteris

19 Coxa, retro surface, Leiobunum negropalpi.
20 Same, proximal aperture.

A, retro articulation of coxa with trochanter. *I*, interarticular membrane

21 Coxa, Theridium tepidariorum, proximal view.
22 Same, distal view.

A, articulation with trochanter
A_1, pro articulation of coxa with thorax
A_2, retro articulation of coxa with thorax
C, crista
I, interarticular membrane
T, sclerite

23 Distal end of trochanter, Theridium tepidariorum. *A*, articulation with coxa.
24 Proximal end of femur, Theridium tepidariorum.

A_1, pro articulation of femur with trochanter
A_2, retro articulation of femur with trochanter

25 Ventral view of trochanter, Leiobunum negropalpi.
26 Same, dorsal view.

A, retro articulation of trochanter with coxa
A_1, dorsal articulation of trochanter
A_2, ventral articulation of trochanter
A_3, pro articulation
C, crista
DL, dorsal lip
VL, ventral lip

27 Retro surface of trochanter, Theridium tepidariorum.
28 Same, distal end.

A, articulation with coxa
A_1, ventral articulation
A_2, dorsal articulation

29 Proximal end of trochanter, Leiobunum negropalpi.

A_1, retro articulation
A_2, pro articulation
DL, dorsal lip
I, interarticular membrane
VL, ventral lip

30 Proximal end of femur, L. negropalpi.

A_1, dorsal articulation. A_2, ventral articulation

31 First two joints of leg, Theridium tepidariorum, pro view.
32 Same, retro view.

A, articulation. *C*, crista. *I*, interarticular membrane.

33 Distal end of trochanter, Leiobunum negropalpi.

A_1, dorsal articulation. A_2, ventral articulation. *I*, interarticular membrane.

AUTOTOMY IN ARACHNIDA
FLORENCE DOWDEN WOOD

PLATE 2

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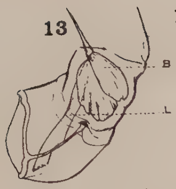

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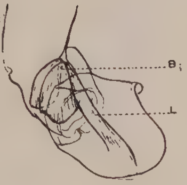

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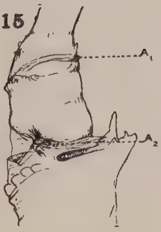

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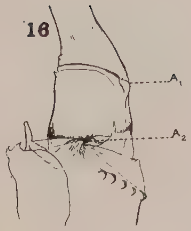

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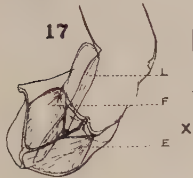

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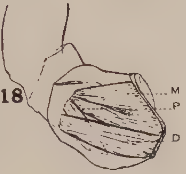

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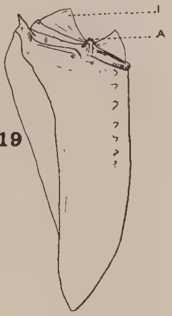

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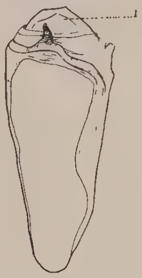

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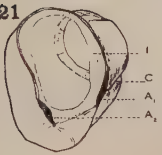

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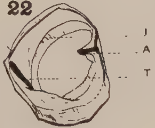

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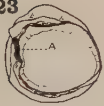

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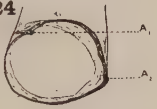

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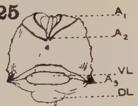

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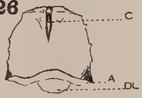

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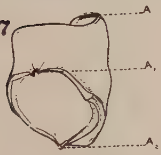

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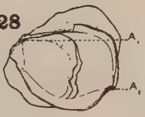

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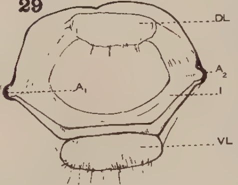

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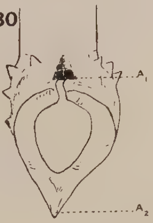

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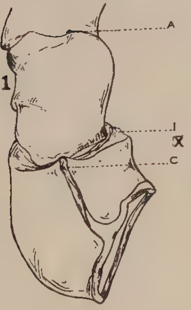

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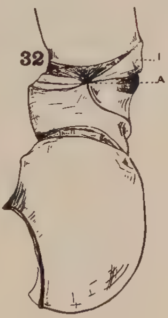

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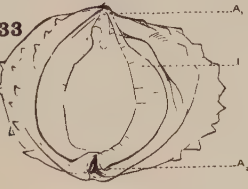


PLATE 3

EXPLANATION OF FIGURES

34 Third right leg of Centrurus carolinianus, pro surface.

35 Same, retro surface.

A, pro articulation between coxa and trochanter
B, pro articulation between trochanter and femur
C, pro articulation between femur and patella
D, pro articulation between patella and tibia
E, ventral articulation between tibia and metatarsus
F, pro articulation between tarsus and metatarsus
G, claws, dorsal side
H, claws, ventral side
I, retro articulation between tarsus and metatarsus
J, dorsal articulation between metatarsus and tibia
K, retro articulation between patella and tibia
L, retro articulation between femur and patella
M, retro articulation between trochanter and femur
N, retro articulation between coxa and trochanter

36 Distal end of patella, Tarantula marginemaculata.

A_1, prodorsal articulation. A_2, retro articulation

37 Proximal end of patella, T. marginemaculata.

A, retro articulation. A_2, pro articulation. *S*, sclerite.

38 Proximal end of tibia, T. marginemaculata.

A_1, dorsal articulation. A_2, retro articulation. *W*, insertion of tibial flexor muscle

39 Proximal end of trochanter, T. marginemaculata.

40 Same, distal end.

A_1, retro articulation A_2, pro articulation A_3, articulation with coxa

41 Proximal end of femur, T. marginemaculata.

A_1, pro articulation
A_2, retro articulation
I, interarticular membrane
Q, attachment for femoral depressor muscle

42 Distal end of femur, T. marginemaculata.

A_1, pro articulation
A_2, retro articulation
F, chitinous flap
I, interarticular membrane

43 Articulations of femur, patella, and tibia, pro view, T. marginemaculata.

A, dorsal articulation
A_1, pro articulation
A_2, prodorsal articulation
S, sclerite

44 Articulations of femur, patella, and tibia, retro view, T. marginemaculata.

A_1, retro articulation
A_2, retrodorsal articulation
I, interarticular membrane

45 Distal end of trochanter, T. marginemaculata.

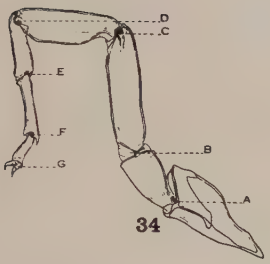
A, articulation (end of crista) *I*, interarticular membrane *S*, sclerite

46 Coxa, pro view, T. marginemaculata. *C*, crista.

47 Coxa and trochanter, T. marginemaculata, pro view.

48 Same, retro view.

A, retro articulation
A_1, pro articulation between trochanter and femur
A_3, pro articulation between coxa and trochanter
C, crista
I, interarticular membrane
Q, attachment for femoral depressor muscles
S, sclerite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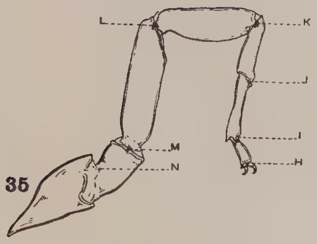
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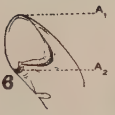
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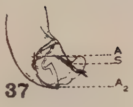
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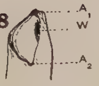
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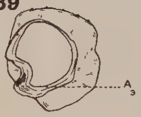
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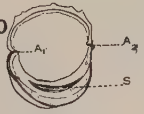
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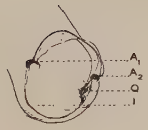
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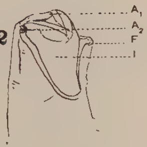
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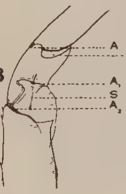
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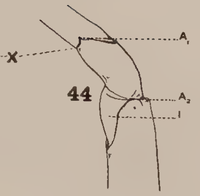
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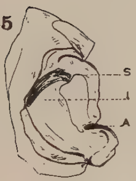
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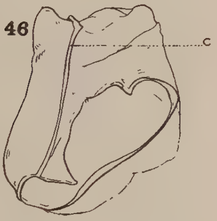
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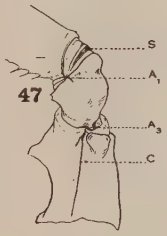
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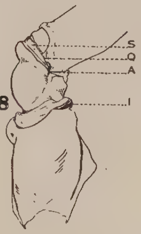
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PLATE 4

EXPLANATION OF FIGURES

49 Transverse section through thorax of Pisaurina mira.

C, coxa
CNS, central nervous system
P, plug formed by blood clot, so-called 'chitinous plug'
PS, pumping stomach

50 Longitudinal section through leg of Lycosa punctulata.

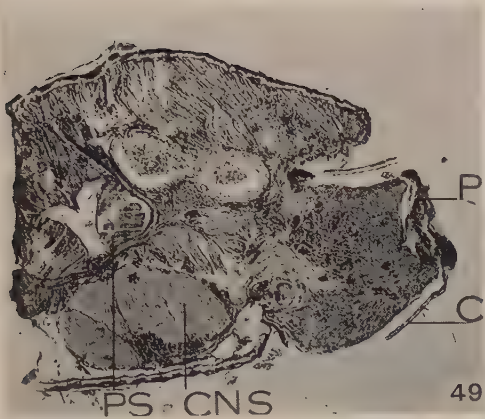
B, m. flexor bilobatus femoris
C, coxa
F, femur
K, m. flexor longus femoris
L, m. flexor longus patellae
N, nerve
T, trochanter
W, weak attachment of trochanter to coxa; it appears as a line of demarcation between the two segments.

51 Longitudinal section through partly autotomized leg of Lycosa punctulata.

B, m. flexor bilobatus femoris
C, coxa
F, femur
L, m. flexor longus patellae
T, trochanter
Z, m. flexor longus femoris, showing severed muscle fiber within the sarcolemma. The muscle is leaving its insertion in the femur.

52 Autotomized leg of Pisaurina mira, longitudinal section.

B, m. flexor bilobatus femoris
F, femur
N, nerve
O, vacancy left by the severing of m. flexor longus femoris
T, trochanter



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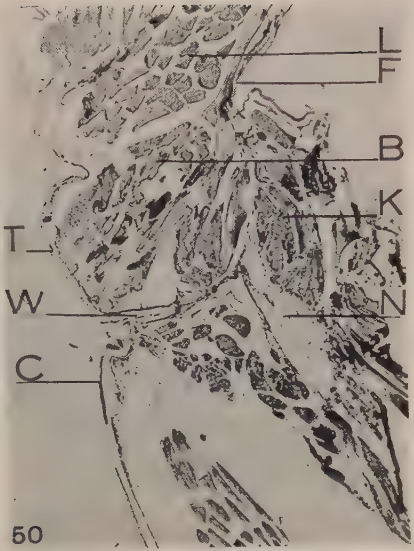

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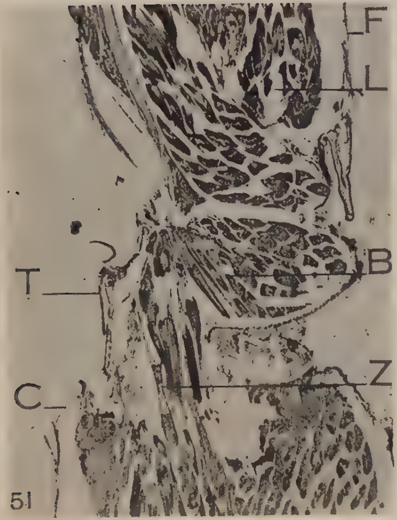

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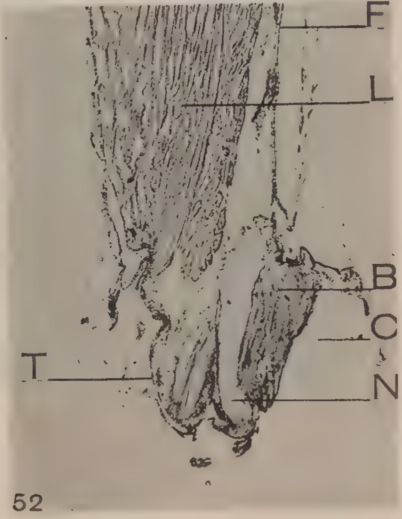

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PLATE 5

EXPLANATION OF FIGURES

53 Longitudinal section through leg of Leiobunum negropalpi.

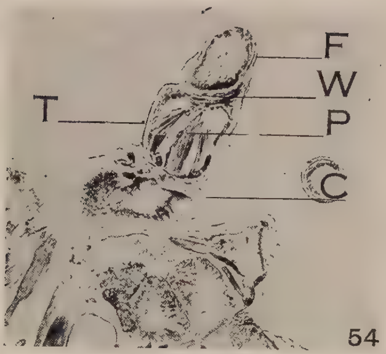
C, coxa
D, m. depressor femoris
F, femur
L, m. levator femoris
T, trochanter
W, weak attachment of trochanter to femur

54 Longitudinal section through thorax and leg of Leiobunum negropalpi.

C, coxa
F, femur
P, m. protractor femoris
T, trochanter
W, weak attachment of femur to trochanter

55 Longitudinal section through leg of Leiobunum negropalpi.

C, coxa
F, femur
N, nerve
R, m. retractor femoris
T, trochanter
W, weak attachment of femur to trochanter



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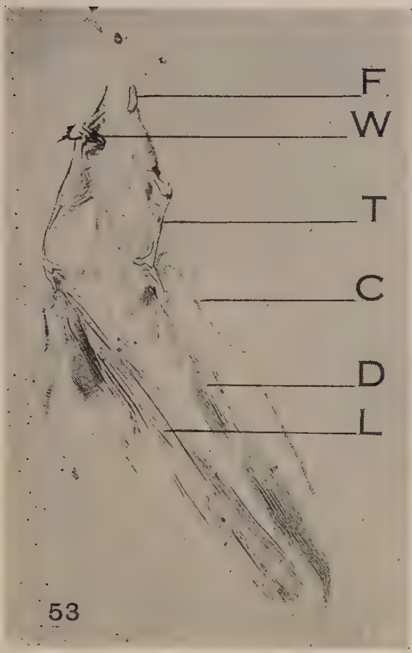

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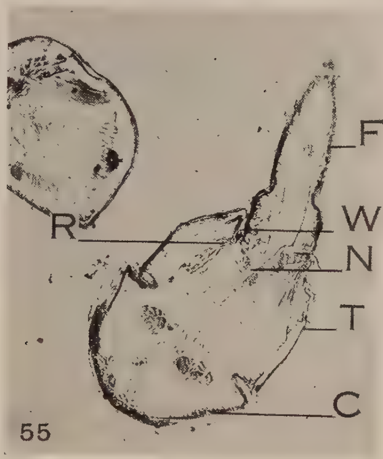

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TARANTULA VERSUS TARANTULA-HAWK: A STUDY IN INSTINCT[1]

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TWO PLATES (FIFTEEN FIGURES)

For years past I have wished to study in detail the encounter between a tarantula and a tarantula-hawk—mode of recognition, methods of attack and of defense, danger to both combatants, relative frequency of failure due to miscarriage of instinct, disparity of size, or inadequacy of weapons. Existing brief descriptions of such encounters and pictures illustrating them are quite insufficient to clear the many problems involved in the study of such a complicated relationship between an insect and a spider. The usual impression is that the danger of being killed is not a negligible factor in the existence of the solitary wasp, which is entirely dependent for its food in its larval stage upon the successful termination of the combat. But if the tarantula is capable of destroying the wasp, then the accounts do not elucidate the reason why she does not do it every time, why does she not kill her mortal enemy outright, as she does in case of molestation by any other insect? Is the spider taken unawares? Is it stung and paralyzed before it has even a chance to assume a defensive attitude? Or is the insect so agile that the clumsy tarantula stands little chance unless she is wide-awake to the danger confronting her? Or is she perhaps deceived in some unaccountable manner and, misled by her senses, permits the wasp to approach unmolested within striking distance, when it is too late to ward off the fatal stab, even though no other living creature is ever admitted within such close range without being immediately pounced upon and destroyed, not even a male of the same species, unless he approaches the female during the breeding season when some mysterious feeling tells the tarantula that the intruder is not an enemy, nor a desirable morsel of food, but a mate and that the hour of reproduction has struck? All these questions may be answered only by experiment and observation in the field and under laboratory conditions. I seized, therefore, the first opportunity which presented itself on my visit to Porto Rico to attack the problem, and while many of the questions remain unanswered and others arose in the progress of the work, the results of the present investigation are sufficiently clear to shed new light on the whole problem.

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[1] A contribution from the University of Porto Rico.

Several species of tarantulae occur in Porto Rico. Their detailed description and a key to their identification will be given in my monograph on ‘‘The Spiders of Porto Rico,’’ which is in progress of preparation. One of these, Avicularia laeta, is not common, although it occurs in various localities and is represented in my collection by four specimens, all females. Another, Cyrtopholis portoricae, is on the contrary, quite common and specimens may be procured in any number by digging them up from their burrows or pulling them out with the aid of a blade of grass or a small ball of beeswax fastened to the end of a fine spring wire or a mandolin A-string. The latter method is more tedious, but, if successful, the tarantula which holds on to the wax with her fangs invariably remains uninjured, while many specimens are killed or injured by the spade even if one is very careful.

Burrows of Cyrtopholis portoricae are common everywhere, on the level ground and in embankments along the roads. The entrance to the burrow is a round hole from an inch to an inch and a half in diameter, unprotected by any web. The burrow itself is tubular, but never straight, and about a foot deep. The size of the tarantula determines the diameter of the burrow. Both sexes make burrows, but males are much less frequent. In December and January, I have captured wandering males on the ground and on the walls of buildings and stone fences, evidently in quest of females. But females seem never to leave their burrows, unless it be at night. The entrance to the burrow is often strewn with the remains of large millipedes common in Porto Rico and forming a large and important part of the tarantula's food.

Cyrtopholis portoricae is not what one would call either a 'hairy' or a 'large' tarantula. Much larger species occur in Hayti, Cuba, Mexico, and Central America; one, a native of Santo Domingo, is found occasionally also in Porto Rico. Nevertheless, Cyrtopholis is by no means a small spider, some females attaining the body length of 2 inches and a spread of legs of 4 inches. The body is heavily built, the legs moderately long and strong. The chelicerae are powerful and the fangs long and hard as steel. The hair clothing the body and the legs is thick and smooth, but not long. The general color above is brown, like that of unsweetened chocolate. The carapace has a distinct grayish-buff marginal stripe. Stripes of the same color are on the dorsal surface of all legs, a pair of them on the femora, patellae, and tibiae, and a very short single stripe at the base of all metatarsi. Below, body and legs are darker, the former being often of a rich velvet black. Like almost all theraphosid spiders, Cyrtopholis his eight eyes in a compact group on a tubercle at the anterior end of the carapace. The sense of sight, however, is very imperfect, for one can move an object to and fro close to the eyes of the creature without in the least disturbing it or producing any sign of perception. The male seems to be somewhat more sensitive to light and at times is aware of a moving object, such as an approaching hand, when he immediately assumes a defensive attitude and even tries to strike. There is no indication of any kind that either the sense of smell or that of hearing is developed to any appreciable degree, or, for that matter, that they are at all present in these spiders. On the other hand, the sense of touch is highly developed in both sexes, the gentlest touch or the slightest breath producing an immediate reaction. If touched with a blade of grass the tarantula strikes it and holds on to it for quite a while. Touched with the end of a glass rod she assumes the defensive attitude, and if again molested, strikes viciously, grabs the rod, and hangs on to it, so that she can be raised in the air from the ground and held in that position for quite a while. When her hunger has been satiated, a milliped is permitted to crawl by or under a leg without being attacked, but at other times even very agile insects, such as some beetles and cockroaches, are instantly pounced upon and killed with lightning speed. So rapid is the attack that it is impossible to see what has taken place. All one hears is a crunching sound and all one sees after the momentary and almost explosive commotion is the tarantula sitting already quite still with the crushed prey in her jaws.

The well-developed claw-tufts at the end of the feet permit Cyrtopholis to climb a vertical pane of glass without difficulty and even with some speed. Yet in captivity she keeps to the ground or, if disturbed, climbs the wall of the cage and occupies a position in the corner under the top. Supplied with food in the shape of live insects and with water, Cyrtopholis is easily kept and does not make any attempt at digging a burrow in the soil. Unless disturbed, her motions are slow and deliberate. Figure 2 shows a large female Cyrtopholis, half natural size, in her normal attitude of progress. As already stated, when touched with a glass rod, Cyrtopholis assumes an attitude of defense and warning. She does this by lifting her four anterior legs, raising her body to an inclined position with the aid of the four posterior legs, and at the same time slowly opening her fangs. Figure 3 shows a somewhat smaller Cyrtopholis female in such an attitude. In this position the spider may remain almost motionless for several minutes. If further disturbed, it will strike suddenly, with great swiftness and force. If left alone, the front legs will gradually come down to the ground and the spider will move away slowly to a quieter place.

The tarantula-hawks, or tarantula-killers, are large digger-wasps belonging to the family Pompilidae among the so-called solitary wasps. All species of digger-wasps specialize in paralyzing spiders or insects as food for the larvae. They are the chief enemies of spiders. Few birds eat spiders, and then only occasionally, not as exclusive diet. Lizards are more dangerous. Some of them eat spiders regularly whenever they manage to find them. They do not go in search of spiders, but simply grab and swallow any moving spider which comes within the range of their vision. On the other hand, solitary wasps are true experts, and it is a well-known fact among arachnologists that it is often easier to find rare species of spiders in mud-daubers' nests than by searching for them in their natural haunts. Pepsis, a genus comprising the largest digger-wasps, specialize in tarantulae.

Five species of Pepsis occur in Porto Rico. All are either dark blue or dark blue with orange wings. They have the appearance of very large hornets and may be seen flying close over the ground or higher up, around flowering bushes. Their motions are slow and deliberate, but they are able to dart away suddenly at great speed, especially when menaced with an insect net which they see well. In capturing them one has to be very careful, as their sting is exceedingly painful.

The species which is more common than the others in the vicinity of Rio Piedras is Pepsis marginata (Palissot de Beauvais). In diffused light the body and legs of this beautiful digger-wasp are almost black and there are black tips to the dull orange wings. But in direct sun rays the body is of a vivid blue color, the legs deep blue, and the wings brilliant orange with black tips. The average size of a female is about 1½ inch. It is not so simple to capture them. They escape by rising to a greater height, but with due patience and agility one may be sure to secure a few specimens. All observations recorded in the following pages were made on three specimens, the first of which was captured in November and the other two in December. It is not possible to keep more than one specimen of Pepsis in the same cage or jar, because they begin immediately to fight with each other in a most vicious manner. On the other hand, in separate cages Pepsis may be kept in good condition for several weeks if properly fed and supplied with sufficient quantity of water. As food, orange marmalade proved to be quite satisfactory. Water was given in an inch-deep glass dish, as a shallower dish is soon filled with dirt by the insect which spends much of her time in walking and digging.

The first observations were made in a cage with a floor space of a square foot and a height of 16 inches. Three sides of this cage were of fine metal wire netting; the fourth, of glass. The top was of the same netting as the sides. About an inch of dirt was placed at the bottom. Later, for the purposes of photography, a small, rectangular aquarium was chosen with metal wire-net top. It was found necessary to use reflected sunlight to secure good negatives, as diffused light requires too long exposures. The cage cannot be placed directly in the sunshine, since that disturbs both the spider and the insect and changes their behavior. It was necessary, therefore, to keep the cage on a table in diffused light, close to the window, and to flood it with sunlight reflected from a mirror at the moment of the combat, which it was desired to record. To get sufficiently large pictures and at the same time to keep the camera sufficiently far from the object to avoid distortion and to obtain a greater depth of focus, a telephoto lens was used. With an aperture of F. 9 and orthochromatic cut-film an exposure of one fiftieth of a second was sufficient. Even so, the number of failures was far greater than the number of successful photographs, as the subject proved from every point of view one of the most difficult to handle.

On being released in the cage, Pepsis[2] flies immediately toward the window and begins to walk up and down the wire netting, tapping it with her feelers and occasionally biting it with great force. After a while she gets accustomed to her new surroundings and begins to walk on all sides of the cage, as well as on its top. Her motion is jerky, but not nervous as in other species of digger-wasps. She cleans herself continuously, now passing her front legs over her antennae, now dragging her hind legs behind her and rubbing them against each other in doing so. Sometimes she walks on the ends of her tibiae of the first pair of legs by bending the tarsi until they are dragged with their dorsal surface on the ground. Sometimes she raises her wings and rubs the sides of the abdomen with the second pair of her legs. The process of cleaning is interrupted only by a close examination of every object which the Pepsis approaches. Although she seems to see objects well, the actual examination is always accomplished by tapping the object with the ends of the antennae. Thus every nook and cranny in the cage is examined, and presently she discovers the tarantula.

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[2] The following account is in the present tense, being an exact reproduction of the journal kept at the time of the observation.

The first encounter is in no way remarkable. The tarantula is sitting in the darkest corner on the ground. True to her nature, she avoids light as far as possible. She sits with her face toward the wall completely unaware of the presence of any other creature in the cage. The Pepsis taps the abdomen of the tarantula from the rear. The tarantula turns round and raises her four front legs in the air in the attitude of defense. The Pepsis seems to be satisfied for the present with the discovery of her prospective victim. She turns sideways and walks lightly away while the tarantula remains for some two or three minutes in her attitude of defense, then slowly lowers her body and legs and rests on the ground.

For several minutes Pepsis walks around the cage, up and down the wall, cleaning herself, nosing around. Occasionally she approaches the tarantula to within the short space of an inch, but turns away as if satisfied with her investigation. After a while she again approaches the tarantula, this time from the side. She taps lightly the legs with her feelers and forces the tarantula to rise in defense. Will the Pepsis leave her again, satisfied with the presence of the tarantula? No, this time she walks deliberately in front of the menacing tarantula. She touches the tips of the legs of the tarantula with her own legs, she taps with her antennae the terrible chelicerae with the fully outstretched fangs. Suddenly she skips under the tarantula and emerges on the other side, walking away as if nothing has happened, while the tarantula continues to stand for a long time in her attitude of defense. Why did she not crush her enemy? Is she afraid? Or has she been satisfied that it is not an enemy? Is she perhaps replete with food and indifferent to excitation? I put a large cockroach into the cage and touch it with a pencil. The unfortunate creature rushes straight toward the tarantula, comes in contact with her, and is pounced upon in a flash. All one hears is the crunching sound as the powerful jaws break up the chitinous skeleton. For a few moments the cockroach wiggles one of its legs, then all motion ceases, only the jaws of the tarantula move. Presently nothing but the wings and pieces of the skeleton remain and the tarantula moves off a couple of inches.

Still a few minutes pass. The pepsis flies with a great noise made by the wings across the cage, alights on the opposite wall and glides down toward the ground. Suddenly, for some reason, she loses her footing on the netting and falls backward on top of the tarantula. The tarantula rears in great rage. By this time the Pepsis has righted herself and walks straight into the jaws of the fierce-looking spider. They stand for a moment face to face, tips of legs to tips of legs. The wasp raises her four wings at right angles above her body and slips head on under the tarantula. The tarantula strikes with force and one hears a loud crunching sound. I think it is all over with the Pepsis. But before the tarantula cools down, her enemy appears behind her, none the worse for the fray, and walks off cleaning her antennae. A fourth encounter the same afternoon gave the same results. By now it was getting dark. Fear that the Pepsis might go to sleep as other wasps do and fall victim of her drowsiness if the tarantula should by chance come in contact with her while wandering at night, decided me to remove the wasp to a large jar with a wide mouth closed by a cork. To prevent the insect from working herself to death in the morning by trying to escape from confinement, I covered the jar with a black cloth.

In the morning I find part of the cork chewed into small particles and the Pepsis somewhat exhausted. I put her into the cage with the tarantula and place a Cresca fig in the hope that it may be taken as food. In a few moments the Pepsis is indeed chewing at it, but soon leaves off. I put a few drops of orange marmalade on top of the fig. The Pepsis returns almost immediately and licks greedily until no marmalade is left. Then she begins to reinvestigate the cage and soon discovers the tarantula. During that day she has twenty-seven encounters with the tarantula in the intervals between which she cleans herself, walks, digs holes in the ground in two places, tries to eat a small piece of cheese which I place on the ground, licks up more marmalade. For a while she works diligently digging a hole about an inch in diameter, that is of about the same diameter as the holes inhabited by tarantulae. Is she preparing the grave for her victim? Solitary wasps do not kill unless they are ready to lay eggs. Nitrogenous food is necessary to induce ants and bees to lay eggs in certain cases. But how is one to find the proper food for the Pepsis?

The work on the hole goes on. The Pepsis grasps pieces of soil with her mandibles, passing the dirt between her legs. The front legs are used in digging. The pieces are sometimes half an inch large, but more commonly smaller. Unfortunately, the soil is not deep and the metal bottom of the cage is soon reached. I purposely did not give much soil, as otherwise the tarantula, too, might dig herself in and observation would become impossible. For the present it is necessary to make as many observations as possible to determine the reason why the tarantula behaves so differently toward all other living creatures as compared with the Pepsis.

The twenty-seven encounters are, however, all more or less of the same type as on the previous day. On two occasions the mix-up was fierce and I was almost sure that I heard distinctly how the tarantula severed the wings of the wasp from the body. But that was only an illusion. The sound undoubtedly came from the crushing of the wings in the jaws of the tarantula, but somehow the wings are no worse for it than before. On two occasions one could see clearly how Pepsis brought the end of her abdomen under the sternum of the tarantula and tried to sting her several times. In the scuffle the tarantula fell on her back, Pepsis walked off, neither was hurt. Toward the evening the Pepsis shows unexpectedly a new mode of behavior. She brings the tarantula to her feet, touches the tips of her feet with the tips of her own feet, taps the jaws of the tarantula with her antennae, raises her wings, and suddenly produces a rather pungent odor. Where did the odor come from and how was it produced? Are there skin-glands situated somewhere on or under the wings? Are they rectal glands or glands of the poison apparatus? It is impossible to decide from the first observation. Meanwhile the Pepsis walks off with the scent trailing behind her. Does the tarantula object to the odor? Or does she like it and being pleased permit the aggressor to escape? Does she notice the odor at all? The question as to whether spiders possess the sense of smell is far from having been settled, but, from what I have observed in nature and under laboratory conditions, I feel more than skeptical toward the school which teaches that they possess such a sense. Yet is it not possible that the sense is attuned to certain odors only and may convey the impression of pleasure or displeasure? One thing only is certain—the tarantula is not aware of the presence of any insect however close to her and however hungry she may be, unless that insect happens to come in contact with her.

On the morning of the third day, having once more placed the two enemies together, I notice that the Pepsis has her left front tarsus in a state of paralysis. She walks on her tibiae, clumsily, apparently with pain. Often she stops and cleans her front legs, both of them, by passing them repeatedly between her mandibles. Has she injured her leg in the early morning in trying to escape from confinement or did the tarantula in her last encounter break a joint without my having noticed it at the time? I am unable to answer this question, but injury notwithstanding, the Pepsis shows no fear of the tarantula. It is true that the encounters are not as frequent as before, but the actions of the Pepsis are as deliberate and apparently the rage of the tarantula does not avail against the quiet assurance of the Pepsis.

The fourth day passes in the same manner. By the end of the day, the tarantula-hawk had, since the beginning of the observations, more than fifty encounters with the tarantula and both showed the same behavior. Once more I put a cockroach into the cage. It walks rapidly toward the tarantula and touches one of her legs with his antennae. The tarantula rises on her hind legs, but almost immediately lowers the front legs again until they touch the ground. The cockroach pushes his way under the belly of the tarantula gently tickling her abdomen from below with his antennae. The tarantula raises her body without lifting her legs until she stands at least an inch and a half over the ground on the tips of her feet. One more touch by the antennae of the cockroach—the tarantula grabs the creature with all her eight legs and kills it in a fraction of a second.

Fearing that the choice of the species of tarantula may have been wrong, I decide, on the fifth morning, to use another species for the experiment. I have a female Avicularia laeta, a distinctly smaller species than Cyrtopholis, but one which exhibits still greater fighting spirit. She is of a chocolate-brown color, rather hairy, and the pads or scopulae on the tarsi and metatarsi of her legs and on the terminal joint of her palpi are so well developed that the ends look tremendously swollen when compared with the rest of the appendages. Of the habits of this species nothing is known. Apparently it does not live in the ground, for I have caught three females, each time in a silk tube built between leaves of the wild pineapple. In captivity the females also refuse to dig in the ground, but construct 6-inch long vertical tubes in the corner of the cage, in which they live sitting head up. The long hairs on the body and legs of this species make it very sensitive to touch. The slightest movement of the air, the gentlest touch immediately startles the spider and the reaction is invariably sudden, quick, and violent.

Put in the cage with the Pepsis, the Avicularia immediately starts to climb up the walls of the cage till she reaches the top, when she crouches in the corner. Dislodged by gentle blowing, she darts sideways halfway across the cage and immediately crouches again in a corner. Once more dislodged by gentle blowing, she jumps down to the ground, runs up another wall of the cage and assumes the same position in a corner. I touch her with a wire at the end of which is a small ball of beeswax. She grabs it viciously and bites at it several times, then moves to another corner.

The Pepsis is meanwhile walking up and down another wall of the cage, occasionally walking on the ground, improving the hole in the ground—the future grave of the tarantula—cleaning herself, nursing her lame foot. Presently she discovers the Avicularia. She walks over her legs and body without in any way producing an effect on the spider. Again she returns, walks over the back of the Avicularia, catches a leg with the claws of her hind leg, pulls at it, and goes away. Avicularia does not respond. She is as motionless as if paralyzed. Eleven times the Pepsis crawls over the tarantula in the same fashion. Now I blow gently at Avicularia and in a bound she crosses the cage to the other corner. She is, then, perfectly sensitive to touch, but for some unaccountable reason does not respond to the touch of the Pepsis or instinctively keeps quiet. For half a day I keep the Avicularia in the same cage with the Pepsis, but as nothing at all happens, I remove her to her own cage and put again the Cyrtopholis with Pepsis. Nothing happens until five o'clock in the afternoon. I engage in a conversation which lasts for a few minutes during which I forget my experiment, although the cage is within 5 feet from where I stand. Something, I do not know what, attracts my attention to the cage. What is that? The 'deed' has been committed and I have not seen it! The tarantula lies helpless on her back and the Pepsis sits on her victim with the sting still in the body of the slain. I watch for further developments, even though the most important moment has escaped my observation. The Pepsis comes off the tarantula to the ground, takes a leg in her mandibles and begins to drag the limp body, stemming her own legs with great force against the ground. It is heavy work. The tarantula must be several times heavier than Pepsis and the ground is rough. In one place Pepsis finds it too difficult to drag the tarantula. She takes another leg in her mandibles and turns the tarantula over on her belly. Again she drags her a few inches. Now she leaves the burden and disappears for a moment in the depression in the ground, which she prepared for the purpose. It is no more than a depression, for the soil is not deep enough. While she is examining the grave I pick up the paralyzed tarantula and examine the body with the aid of a lens. White blood is coming out of a little prick-wound in the articulation membrane between the sternum, lip, maxillae, and first coxae on the right side of the body. So that is where she has been stabbed. The sternum is too hard for a sting to pierce it, yet under the sternum the great thoracic ganglion is situated. The Pepsis has reached it in a slanting direction from the nearest vulnerable point.

I bring more soil and put it into the cage. Not to injure the Pepsis, I try to drive her out of the grave with the end of my forceps. She becomes angry and buzzes loudly with her wings. Suddenly the familiar odor fills the cage. So the production of this odor must be a sign of anger, perhaps of warning. I leave her in peace, heaping up the soil elsewhere so that now the grave is indeed quite deep, with an irregular wide mouth. I return the body of the tarantula to the cage, placing it on its back in the middle of the cage. Immediately the Pepsis comes out of the grave and begins to drag the body toward it. A few resting moments, a few exertions, with the legs and abdomen of Pepsis stemmed against the ground, once again the spider is turned over on her belly and dragged face down into the grave. Meanwhile the tropical twilight rapidly fades, it becomes dark, and the Pepsis rests in the grave pressing her body against the body of her paralyzed victim.

About nine in the evening she decides to change the place of burial. She drags out the tarantula and pulls her under great difficulties across the cage to another corner, in which she buries the body. In the morning I try to find the tarantula, but all outward traces of dug up soil are obliterated. After having removed almost half of the soil from the entire surface I finally expose the tarantula neatly buried in a hole in the ground some 8 inches from the place where she was buried in the evening. The final grave is just big enough to hold the occupant with her legs drawn close against her body. An egg, about 4.7 mm. long, is attached to the side (fig. 15). The egg is of the type common to wasps and bees, but considerably larger and translucent white. I remove the tarantula, carefully dust away the dirt, and weigh the body. It weighs exactly 8.2 grams. But the creature is not quite dead. Occasionally a leg twitches. There is still life in the tarantula. In fact, she is still sensitive to touch. The leg reacts to gentle blowing from my lips. Are only the motor centers paralyzed, and does the tarantula feel pain when her flesh is attacked by the larva of Pepsis, but remains unable to move?

On the morning of the nineteenth day of November I put a smaller female Cyrtopholis in the cage with the Pepsis. It is a new cage, the soil has been purposely made smooth and hard. Less than thirty-six hours have elapsed since the Pepsis has paralyzed the first victim. The new tarantula has just been dug up from a hole in the ground. She is lively and angry. She walks up the side of the cage and is not to be persuaded by any means to descend to the ground. Time and again I drive her down, but the next moment she climbs up again. I desist and decide to watch. A little later I am, indeed, fortunate enough to witness the Pepsis in the act of killing its prey, and a very interesting and terrible sight it is.

The Pepsis comes deliberately to the tarantula on the side of the cage and drives her down to the ground. Next moment she closes in on her victim in the manner already described and bending her abdomen under the venter of the tarantula introduces the sting between the third and fourth right coxae close to the sternum. The tarantula struggles violently and rolls with the Pepsis over and over on the ground. After a few struggles the Pepsis lets go her hold on the tarantula, walks off a couple of paces, turns and comes directly toward the jaws of the tarantula. Without the slightest hesitation she slips under the tarantula which raises as high as she can on all her legs. The Pepsis grabs the fourth left leg with her mandibles. The tarantula tries to bite her enemy, but the Pepsis holds her off by pressing her feet against the feet of the tarantula, while at the same time continuing her hold on the fourth leg with her mandibles. Meanwhile she bends her abdomen and searches for the place to pierce with her sting. Now she finds it. It is the same place as in the first specimen, that is the articulation membrane between maxilla, first leg, sternum, and lip. In a few seconds the tarantula is paralyzed. The position of the two is very remarkable. The tarantula sits in her normal way, but the Pepsis lies on her right side, head toward the posterior end of the tarantula, sting in the place mentioned. After at least half a minute the Pepsis withdraws her sting and walks off. The tarantula remains motionless. Presently one leg of the tarantula moves. The Pepsis returns, climbs on the tarantula, inserts her sting between the sternum and the third coxa and holds it there for about a minute. Now she goes to dig a grave, but the soil is hard and all her attempts are without avail. Finally she returns to the tarantula and drags the body into a corner. What has she in mind? In the corner is a little glass dish with water, cylindrical in shape, an inch in diameter and a little over half an inch deep. Into that dish the Pepsis stuffs the body as far as it will go. I take pity on the Pepsis and with the end of a penholder make a deep hole in the ground in the center of the cage. At the same time I remove the tarantula from the glass dish and put her close to the hole. The Pepsis discovers the tarantula right away and once more starts to drag her, but this time discovers the hole, leaves the tarantula and begins to dig furiously. Soon the hole is deep and wide enough to permit the Pepsis to disappear in it completely, only to stick out from time to time the posterior legs and end of the abdomen, at the same time throwing dirt with force away from the hole from under her belly. While she is thus occupied I remove the paralyzed tarantula before an egg has been laid, so as to ascertain whether the paralyzed creature may recover with time, and, if so, how soon, or, if not, how long she will remain in the paralyzed condition before she dies. In her place I put into the cage a male of the same species, dug up from a hole the same afternoon. He is very lively and angry and apparently sees objects approaching him from above, for on two occasions he tried to strike at my hand as I was moving it toward him to cover him up with an aluminum cup. In the cage he runs up the wall of the cage immediately and cannot be driven down. The Pepsis discovers him soon. She deliberately grapples with him, both falling together to the ground. The tarantula lies motionless on his back. The Pepsis leaves him. As soon as she is gone, the tarantula jumps up and runs up the side of the cage. He was playing possum! Nothing happens that day, nor the next day. In the evening at about 5.30 P.M. the Pepsis disappears into her hole and does not reappear again until morning. She went evidently to rest there. Next morning I return the male tarantula to the cage with the Pepsis. The latter immediately comes toward him and climbs over the astonished and threatening tarantula. Next she climbs under him. Then she pulls him by his leg. No attempt on the part of the tarantula to defend himself by striking, only by raising his body and handling the Pepsis with his four front legs. The Pepsis walks away. The tarantula runs up the wall. Immediately Pepsis returns and grapples viciously with the tarantula, bringing him down to the ground. Here they separate. Neither of them has been injured. The Pepsis goes to the dish with the marmalade and sucks it for quite a while. Meanwhile the tarantula has again climbed the wall. For a long time the Pepsis has no encounter whatsoever with the tarantula. After a while I have to leave the room for four hours. When I return the gruesome deed has been done. The tarantula has disappeared and the grave also. The soil is neatly leveled, and were it not for the fact that the deeper layer of the soil is clay, the surface dark humus, and that the grave was deep enough to go to the bottom of the cage so that some of the clay was excavated and used afterward to cover up the grave, no trace of the latter could have been found. The Pepsis is, in fact, as skilled in removing all traces of her deed as she is in committing it, even though her left front leg still remains maimed and practically useless, presenting to a great extent an impediment to the proper functioning of the other limbs and organs.

So the male tarantula has been killed as ingloriously as the female, after numerous encounters in which he was perfectly aware of the presence and of the aggressive character of the Pepsis and showed clear signs of fear, while she was not only free from all fear, but provocative—one would almost like to say, insulting. Then, since both sexes behaved more or less in the same manner, it is not likely that the Pepsis in any way deceives her victim into the belief that she is the mate, nor that she is a friend. Instinctively, the tarantula realizes her own helplessness toward the Pepsis and fails in such methods of defense as a swift attack—the only method which could be of any avail here, but which is not in the character of a spider of her family.

Moreover, the Pepsis has the advantage over the tarantula in the possession of good senses, especially of a fine sense of sight. She discovers the tarantula in the cage in a few moments and never loses sight of her, as the following record shows. At 9.37 A.M., a Cyrtopholis is placed in the cage with the Pepsis. The latter is busy in walking rapidly up and down the sides of the cage and to and fro on the ground. At 9.39, the Pepsis flies above the tarantula, examining the latter. At 9.42, she walks by the tarantula and deliberately steps on the latter's 'toes.' At 9.45, she walks across one leg of the tarantula without any response from the latter. At 9.48, she comes from the rear and touches the abdomen of the tarantula, then goes to the grave and does some digging. At 10.15, she goes to the dish with the marmalade and begins eating it, contemptuously turning her back on the tarantula, which sits within less than an inch from her. At 10.25, she is still engaged in eating the marmalade when with one of her hind legs she catches a leg of the tarantula. The latter moves that and another leg away, but does not leave the spot on which she sits.

Does the Pepsis specialize in Cyrtopholis or does she not despise other species as well? Once more I place in her cage an Avicularia laeta. It is the same female with which I have already experimented. In a few seconds she runs up the side of the cage and crouches in an upper corner. The Pepsis discovers her in a few moments, walks deliberately toward her, touches her with the antennae, climbs over her, dives under her, and reappears at the other end. The Avicularia responds but indifferently. Several times the Pepsis repeats her examination, but shows no desire to attack. Three days and nights the two are left in the same cage and nothing happens. I remove the Avicularia and place a Cyrtopholis in the cage. In less than half a minute the Pepsis attacks her, takes a leg in her mandibles and, holding the tarantula at a distance by means of her own tarsi stemming the legs of the tarantula, inserts her sting into the soft membrane between the third and fourth coxae. A brief struggle ensues, after which the tarantula is partly but not completely paralyzed. The Pepsis leaves her for a few moments, discovers motion in the legs of her victim, returns, inserts her sting after considerable search in the membrane between the maxilla and first coxa, holds it there for a minute, and leaves then the completely paralyzed creature for a few seconds, returning almost immediately to lick up the blood oozing out of the wound. For some reason she does not dig a hole and leaves the tarantula lying on the ground. In the afternoon of the same day I place another Cyrtopholis with the wasp. It is a small and very active specimen, ready to fight any object brought in contact with her. The Pepsis examines her from a distance, walks toward her, and dives straight under her chest. Emerging at the rear she grasps one of the hind legs of the tarantula, and the latter runs up the side of the cage dragging the wasp behind her. At the top of the cage the Pepsis forces the tarantula to take a defensive attitude, in consequence of which both fall on the ground. A fierce battle ensues. The tarantula enfolds the Pepsis in her eight legs and strikes repeatedly and viciously with her fangs. The wasp is altogether invisible in the grasp of the tarantula, which, though a small specimen as tarantulae go, is still considerably larger than the Pepsis. One can hear the loud crunching sound as the tarantula tries to crush her adversary with her fangs. Surely, this is the end of the Pepsis! But no. Perhaps a minute elapses, the struggling coil becomes quiet, the tarantula lies paralyzed and the triumphant Pepsis climbs out from under her victim, none the worse for the fray. She brushes her entire body by dragging herself over the ground, so that again for a moment the impression is produced that, after all, the Pepsis was badly hurt. But in another moment she has finished her toilet, rises to her feet in a normal posture, turns around, and notices that the Cyrtopholis shows distinct signs of life. Two of the legs are twitching rhythmically and considerably. The Pepsis approaches the tarantula, examines her, climbs on her chest—the tarantula, as usual after the encounter, lying on her back—takes a leg in her mandibles, searches for the spot between the maxilla and first coxa, finds it finally, inserts the sting, and continues stinging for three solid minutes, all the while working the sting. When this act is finished, the Pepsis withdraws her sting, turns, and begins to lap up the blood which is coming rather rapidly out of the wound. Now the Pepsis leaves the body and digs a grave. Into that grave she drags her victim and stays with it in the grave for quite a considerable length of time. Finally she reappears and fills the grave until nothing shows the place.

Again I place the Avicularia for two days without any results. Removing the Avicularia, I place a 'guava,' i.e., a species of pedipalpi or whip-scorpions. The guava, however, has no whip. Its scientific name is Tarantula palmata—an unfortunate confusion of names, since it has nothing to do either with the European spider known there as tarantula or with the American spiders commonly called tarantulae. It is a large specimen, 1¼ inches in length, with perfectly flat body adapted to life in crevices of rocks. In front it has a pair of dangerous-looking grasping appendages, longer than the body and provided with sharp, long spines. The legs are thin and several inches long, but of very hard chitin. The second pair is modified into feelers, thin and exceedingly long, so that the span between the tips of the two measures 16 inches when they are stretched out. Usually, however, the creature holds its feelers bent in a peculiar fashion and ready to respond to the slightest touch. When scared, she runs swiftly sideways, crab fashion. The natives are much afraid of the guava, saying that her bite is very poisonous. There are poison glands connected with the short and scarcely noticeable mandibles, but as no experiments have ever been made, it is not safe to assume that the poison is virulent or in any way affecting any creature except the natural prey. For several days Pepsis remains with the guava. She examines the latter and occasionally walks over or under her, forcing her to be continually on her guard, with raised feelers. But nothing further happens. The two do not molest each other, do not even stage a sham-fight or show the slightest desire to attack. By this time the Pepsis has three of her legs in an injured state, due to constant running up and down the screen wall of the cage and digging in the ground. Is she still capable to meet a tarantula in such a state, with only three good legs—the first right leg and the two hind legs? I remove the guava and put a medium-size Cyrtopholis in the cage. In a few moments the wasp attacks the tarantula. She takes a front leg in her mandibles, but has a difficulty in holding the other legs at a distance with her own mutilated leg lacking the tarsi. Consequently, she inserts the sting in the nearest accessible soft spot which happens to be between the third and fourth coxae. The tarantula struggles and tries to bite, to crush her enemy, but succumbs to the invulnerable enemy and is soon buried. Eight tarantulae killed one after another with endless chances to defend themselves if the defense were at all possible. Fortune brings into my possession another specimen of Pepsis, one without any injury. So I preserve the first one in alcohol, having first killed her in cyanide and carefully weighed her to compare with the weight of the first tarantula. The Pepsis weighs only 1.065 grams, one-eighth the weight of the tarantula. Imagine the strength of the insect, which not only kills an enemy so many times heavier than itself, but digs a hole for it in about twenty minutes several times longer than the tarantula, drags the body by its legs a distance of 6 or 8 inches, turns it over, drags it into the hole which is scarcely wide enough to admit one of them. In one case I have seen the Pepsis drag the tarantula for some unaccountable reason up the screen wall of the cage to a height of about 2 inches above the ground before she dropped it.

Does the paralyzed tarantula ever recover from her paralysis, and if so can she get out of her grave? I have found in early spring perfectly live Epeira cornuta in cells of mud-daubing wasps in Connecticut, where they have been placed by the wasp, which failed, however, for some reason to lay an egg. When released, the spiders behaved quite normally and built orbwebs of the usual type. They must have spent at least six months, perhaps eight months, in confinement, the mud of the perfectly sealed cell preventing complete drying up. I have found Epeira cornuta living in abandoned cells from which the wasp has emerged after metamorphosis, thus proving that the spider has no instinctive fear of a cell which served as tomb for other individuals of its own species. I doubt if the Epeira, fortunate enough to have escaped death by being eaten alive, yet can escape death from hunger and thirst except by accident when something happens to break the cell, for the mud is hard and, although it is brittle, the orb-weaving spiders are not fitted out by nature to dig and would be therefore unable to make an opening in the wall of the cell to effect escape. With the tarantula it is different. She is an expert digger, making deep holes in the hardest ground. If she recovered her faculties and found herself in an excavation in the ground, she could easily dig her way out. But could she do anything if she were prevented from using her jaws and legs? The wasp is canny. She packs the soil until it so invests the body and legs of the tarantula that even in case of recovery the unfortunate creature could not move a single limb. I have unearthed a number of tarantulae stung and buried in the laboratory. They were invariably so masterfully packed that even the slightest motion is out of the question. Yet as soon as one removes the soil packed over a leg, that leg contracts and moves. And when the spider has been taken out of its grave every leg is showing contractility and sensitiveness to the breath of the experimenter's mouth. Thus the soil will hold the tarantula even if she should recover, assuring food, undisturbed, to the larva of the Pepsis. Perhaps the most amazing thing about the state of paralysis in which the tarantula is thrown by the sting almost instantaneously is this retention of response to the motion of the air while the heart ceases to beat and remains motionless for several weeks. Even at present, i.e., at the time of this writing, I have a tarantula which is already five weeks in a state of paralysis; her heart does not beat, yet her legs contract when I blow at it ever so lightly. In another specimen an injection of a weak solution of adrenalin restored the heart beat for a couple of hours. Later the heart stopped beating again, yet sensitiveness remained. The tarantula, which was paralyzed November 18th, was kept under observation until the last day of January, when unforeseen circumstances forced me to discontinue the work. Gradually she recovered sufficiently to be able to move a few inches when disturbed. During all this time she had been unable either to eat or to drink, but received an occasional bath and was kept in a jar with a glass cover, to prevent too great a loss of water on her part. Occasionally defecation occurred and the excrements were of the normal color and appearance, but the heart did not beat. My expectation was that with the return of the heart beat, recovery would be complete, but the discontinuance of the observation prevented me from finding an answer to this question.

Since the observations recorded in the preceding pages were made I have been able to make a great many additional observations and numerous photographs of the encounters. They all clearly show two things: that, in a general way, both the tarantula and the wasp behave each time in the same manner, but that in details of the encounter the wasp shows remarkable independence of varied conditions, bordering almost on intelligence. It is unnecessary to give all these observations in extenso. A brief summary should be sufficient.

Pepsis shows no fear of man or beast. Her actions are deliberate. In case of danger or when angered, she raises her wings and at the same time produces a pungent odor either in self-protection or as a warning.

In searching for her victim she uses her sense of sight, and not of smell, as otherwise she would not subject to closer inspection other species of Arachnida. Yet I have seen Pepsis examining besides Cyrtopholis, also Avicularia, Heteropoda venatoria (the so-called huntsman spider), and Tarantula palmata (a pedipalp). In nature she finds the burrow of Cyrtopholis by flying over a field and examining it from above at a short distance. In captivity she notices her prey first, whether moving or not, and proceeds then to closer examination.

The act of examination is consequent upon the finding of a large spider and not dependent upon the state of the ovary, since Pepsis frequently examines tarantulae at a time when she is not capable of laying an egg, as, for example, shortly after having laid one. Usually two or three days have to elapse before another egg can be laid.

The act of ascertaining whether it is Cyrtopholis or some other kind of Arachnid is accomplished by touch. For this purpose Pepsis taps the spider with her antennae, climbs on its back (fig. 5), or slips under its belly, regardless of whether the spider has been taken unawares or is on its guard. The behavior of Pepsis shows perfect confidence and absolute disregard of danger. Since killing is governed by one of the two factors, condition of the ovary or forced encounter when combat is inevitable, the number of encounters which result in no attempt on the part of Pepsis to paralyze her prospective victim, even if the latter is sufficiently irritated to start a fight, is much greater than the number of fatal encounters. Altogether I have witnessed over two hundred encounters, many fatal to the tarantulae, but not one in any way resulting in an injury to Pepsis. One of the three specimens of Pepsis was finally placed for three weeks in the same cage with an Avicularia. The latter stayed always in her silk tube and remained therefore inaccessible to the Pepsis. At the expiration of three weeks, I found the remains of the Pepsis one morning showing that in the night the Avicularia had left her silk home and attacked the wasp successfully. But in nature Pepsis has no chance whatsoever of being attacked by an Avicularia, on account of the difference in their mode of life, and besides it is probable that the wasp was attacked in her sleep, quite unprepared for any defense. Cyrtopholis is the only species used as food for the future larva of Pepsis. It is therefore the only species attacked by Pepsis.

When the time has come for Pepsis to provide a tarantula for an egg which is about ready to be laid, she goes resolutely toward her prey, approaching it from any direction that may be most convenient—from in front, from the rear, or from one side—and, rapidly tapping a leg of the tarantula with her antennae, suddenly grasps it with her mandibles. Usually it is either the fourth or the first leg. During the struggle which ensues and in the course of which the tarantula sometimes succeeds in freeing herself and attempting an escape, but never in inflicting any injury upon the assailant, the wasp firmly holds on to the leg, protects herself from the danger of being crushed by the powerful jaws of the tarantula by stemming her own feet against the feet of her victim, and bending her abdomen begins to search for the articulation membrane between the sternum and the carapace (figs. 6 to 9). There is no special point at which the sting is introduced, it being largely a matter of chance and relative position and proportions of the combatants. Nevertheless, the indications are that the poison acts quickest when the sting is inserted at the anterior end of the sternum. Paralysis results within a second or two, but the wasp continues to sting for half a minute or more.

Having withdrawn her sting, the Pepsis drags herself along the ground (fig. 12), apparently in an endeavor to clean herself. In the photograph we see the paralyzed tarantula lying on her back with a drop of blood oozing out of the wound. The next step in cleaning herself is accomplished by rubbing the hind legs against each other (fig. 11).

A few moments later, the wasp returns to the tarantula and licks greedily the blood from the wound (figs. 13 and 14). If a leg of the tarantula happens still to be twitching, the wasp takes it again in her mandibles and inserts the sting into the articulation membrane as in the first instance. This time, however (fig. 10), the wasp continues to sting for a considerably longer time, and, on having finished, sometimes drags herself again along the ground, at other times omits this performance.

For a while after killing her victim, the wasp seems to be able to recognize it. If one removes it and gives her another tarantula paralyzed a few days before, she becomes uneasy and sometimes desists from her intention to bury her prey. If one puts a live tarantula in place of the one just slain, the wasp immediately attacks and paralyzes her. The impression is as if the wasp mistook the new tarantula for the slain one come to life and acted in the same manner as when on examining a paralyzed tarantula she discovers a leg still twitching. Certainly, she continues to work her sting on the substitute much longer than she does in the first instance. If one places several paralyzed tarantulae on the ground while the wasp is in the grave inspecting it and therefore out of sight, on reappearing the wasp shows considerable confusion. She seems to be able to discriminate between two and at most three specimens, one of which was just paralyzed, but when five paralyzed tarantulae are placed on the ground, as I did on two occasions, the attempt to find the recent victim was given up by the wasp.

The wasp drags the paralyzed tarantula into her grave and then remains there for a considerable length of time, evidently needed for laying an egg and for carefully packing down the soil afterward. On emerging finally from the grave, the wasp removes all traces of her work, by filling the mouth of the grave with soil and leveling the ground. In a few minutes the entrance to the grave cannot be found any more. It may be added that in captivity the wasp sometimes again digs up the buried tarantula and dismembers it. The reason for this action is not clear.

Neither Cyrtopholis, which is the only species used by Pepsis as food for her larvae, nor Avicularia, nor even Tarantula palmata respond to an encounter with Pepsis in the same manner as they do in the case of other insects. They permit Pepsis to walk over or under them and try to strike only when the wasp becomes too aggressive. Cyrtopholis is quite unable to defend herself and invariably succumbs. Underground she remains in a paralyzed state for many weeks.

But if the tarantula is so helpless in an actual encounter with Pepsis, what other protection does she find in nature against her enemy and why are the tarantulae not finally exterminated? One answer is that Pepsis lays not nearly as many eggs as the tarantula and that the latter has apparently no other enemies. Another reason presented itself to my mind when I made some experiments out of doors. On one occasion I took a Pepsis in an insect net and placed the latter over the mouth of a large Cyrtopholis burrow. Immediately the Pepsis disappeared in the burrow and remained there an entire hour. When I have almost despaired of seeing the wasp again and attributed the disappearance of the wasp to a fatal outcome of the underground battle, Pepsis suddenly emerged in a perfect condition. Having placed her in a jar to prevent her escape, I proceeded to examine the burrow with the aid of a spade. At first quite wide, the burrow soon became much smaller in diameter. Next I found a hind leg which was quite recently torn off and still had blood oozing out of the wound. Finally, at the end of the burrow I discovered the tarantula sitting with her head toward the end of the burrow and her abdomen completely filling out the space. She was holding on to the wall of the burrow with such force that it took some trouble to remove her. Far from being paralyzed, the tarantula was so enraged on having been dug up that she tried to strike. Now the Pepsis is unable to pierce the skin of the abdomen, as I had repeatedly occasion to observe in the laboratory. So the tarantula is safe when nothing but the end of her abdomen is exposed to her enemy, because there is no space left between the abdomen and the wall of the burrow for the Pepsis to get by and thus to reach the vulnerable articulation membrane. The tarantula saves her life by plugging her burrow with her own body, perhaps at the expense of a leg, but that damage is easily repaired through regeneration.

University of Porto Rico,
January 28, 1926

PLATE 1

EXPLANATION OF FIGURES

1 Pepsis marginata walking on the ground in the cage.

2 Cyrtopholis portoricae female, walking slowly on the ground.

3 Same female as in preceding figure in the attitude of defense.

4 Cyrtopholis portoricae male, standing erect after a Pepsis has passed under its belly.

5 Pepsis marginata examining a Cyrtopholis portoricae with the object of finding out whether it is the required species, a few minutes before the attack.

6 Cyrtopholis portoricae paralyzed by Pepsis marginata and lying motionless on her back. The wasp is seen still inserting her sting into the soft membrane between the fourth coxae. She is holding the third leg of the spider in her mandibles.

7 The same wasp shown in the act of stinging another specimen of Cyrtopholis. The photograph shows plainly how the wasp holds a leg of the spider in her mandibles.

8 Another photograph of the same wasp stinging a male Cyrtopholis.

9 The same wasp stinging another male Cyrtopholis. The photograph shows that the wasp holds the spider, in this case, by the coxa of the first leg.

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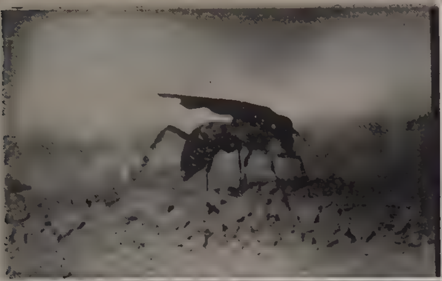

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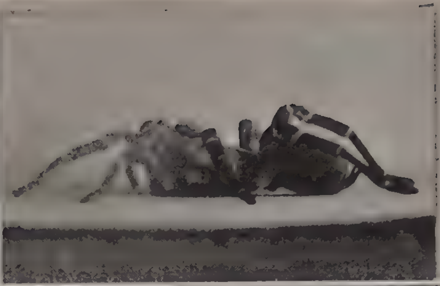

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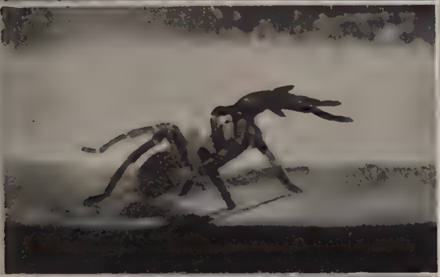

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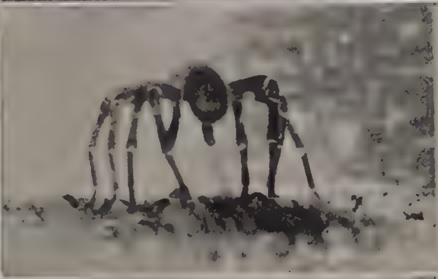

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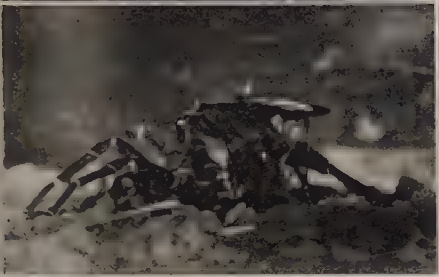

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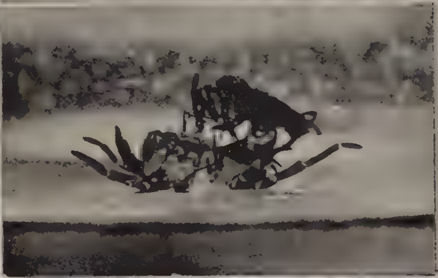

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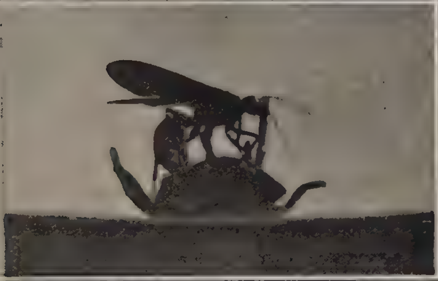

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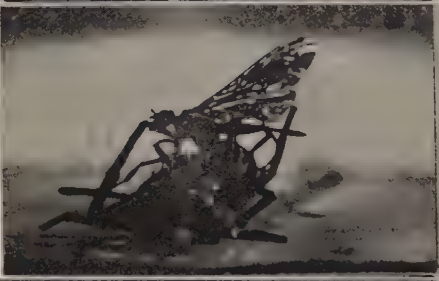

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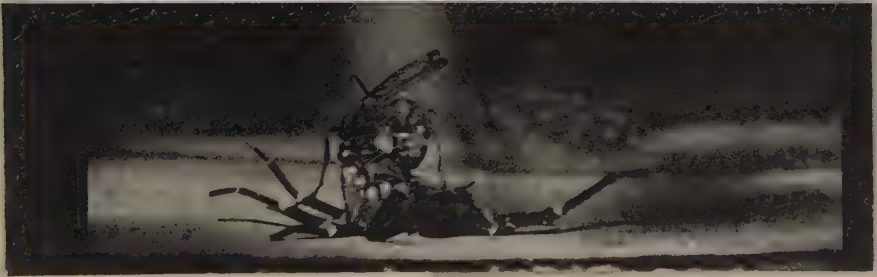

PLATE 2

EXPLANATION OF FIGURES

10 Pepsis marginata stinging a Cyrtopholis female for the second time, after having discovered that one of the spider's legs was still twitching. One can see a drop of blood oozing out of the first wound inflicted by the wasp in the soft membrane at the anterior end of the sternum.

11 Pepsis marginata cleaning her hind legs after having paralyzed a tarantula.

12 The same wasp and tarantula as in figure 10, but a few minutes earlier. The tarantula is shown lying paralyzed on her back with a drop of blood oozing out of the wound, while the wasp cleans herself by dragging her body on the ground.

13 Pepsis marginata licking the blood oozing out of the wound after having paralyzed the tarantula. This is the same wasp and spider as shown in figure 8.

14 Pepsis marginata licking the blood oozing out of the paralyzed tarantula. The same wasp and spider as shown in figure 7.

15 An exposed 'grave' of the tarantula, with the egg of Pepsis marginata laid on the abdomen of the spider. The photograph shows that there is no cavity surrounding the spider and that the original burrow was filled up completely with soil.

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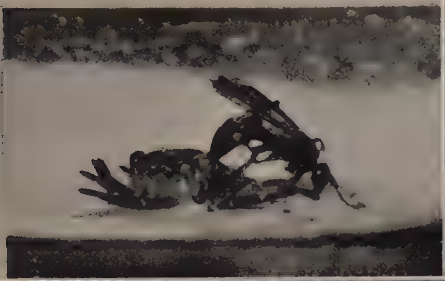

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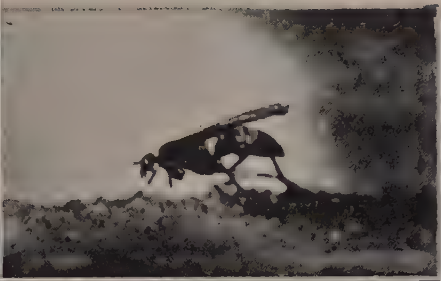

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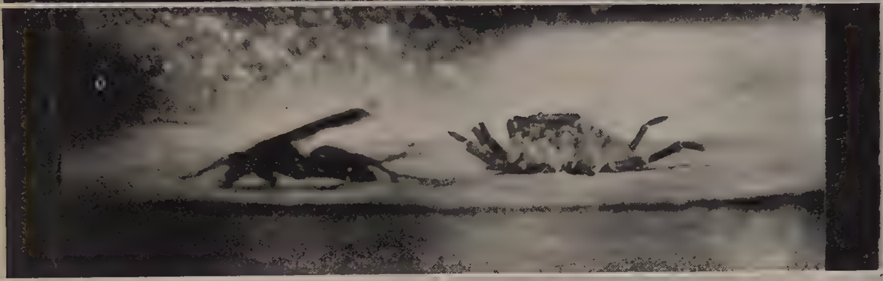

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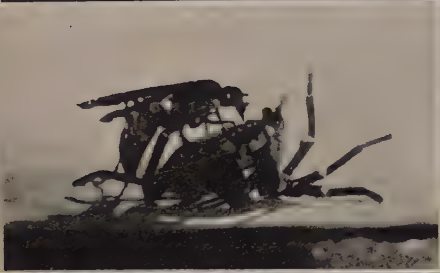

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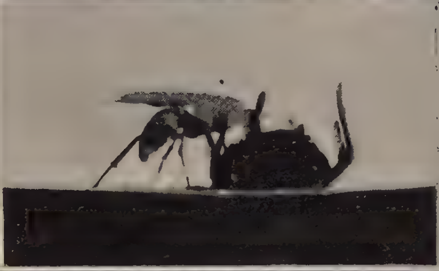

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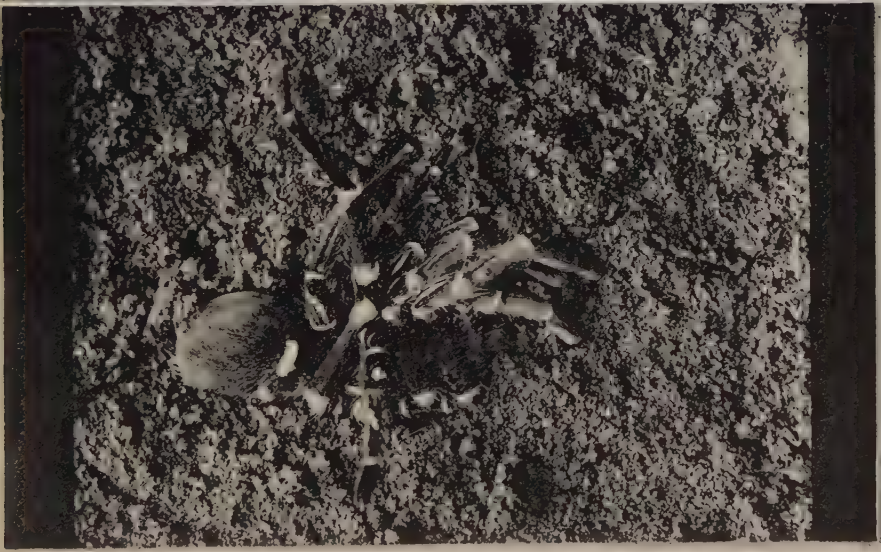

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ELEVEN THOUSAND GENERATIONS OF PARAMECIUM

By LORANDE LOSS WOODRUFF

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CAN unicellular animals reproduce indefinitely without recourse to fertilization? This was the question to which an answer was sought when the experiment to be summarized was begun nineteen years ago. The consensus of opinion at that time, based chiefly on the work of Bütschli, Maupas, Hertwig, and Calkins, was that fertilization is a *sine qua non* in the life history of the Infusoria (1). The conclusion usually, and I believe rightly, drawn from the data available today, largely from the following experiment, is that fertilization plays no indispensible rôle in the life history of Infusoria when subjected to entirely favorable environmental conditions (2). This conclusion, it may be noted, does not deny that fertilization has a stimulating function.

On May 1st, 1907, a pedigree culture of *Paramecium aurelia* was started with a "wild" individual and has been maintained by the isolation of certain of its progeny practically every day, with the exception noted below. The number of divisions has been recorded at each isolation and has afforded a continuous series of data for the study of the reproductive activity of the culture. Throughout the experiment some of the animals discarded from the lines at the daily isolations have been fixed, stained, and mounted as permanent preparations for the study of the cytological changes during the life history. The culture medium for the first eight months of the work consisted of infusions of hay and fresh grass, but from February, 1908, to the present time various materials collected from ponds, swamps, etc., have been employed. The infusions have invariably been thoroughly boiled to prevent the contamination of the pure culture with foreign strains of *Paramecium*. The possibility of conjugation occurring in the culture has been precluded by the almost daily isolation of the products of division. In short, the animals of the culture today are direct lineal descendants, without fertilization, of the single animal isolated in 1907 (3).

In this manner the pedigree culture was carried for eight years, during which 5071 generations were attained, and then (May 1, 1915), the experiment was considered *formally* closed with the statement that the organisms of the present generation are in as normal morphological and physiological condition as the original individual isolated to initiate the culture. The study has indicated that under favorable environmental conditions the protoplasm of the progenitor of the culture possessed (at least) the potentiality to produce similar cells to the number represented by 2 raised to the 5071st power, or a volume of protoplasm greatly exceeding 10^{1000} times the volume of the Earth. I believe this result indicates beyond question that the protoplasm of a single cell may be self sufficient to reproduce itself indefinitely, under favorable conditions, without recourse to fertilization (4).

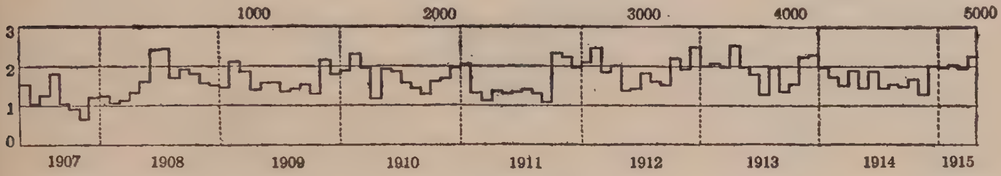
However, with this conclusion secure,

TABLE I

Record of the number of generations attained during each year of the life of the culture from May 1, 1907 to May 1, 1915

YEAR	GENERATIONS	DAILY AVERAGE DIVISION RATE
1	452	1.24
2	690	1.89
3	613	1.68
4	612	1.67
5	662	1.81
6	692	1.89
7	671	1.84
8	679	1.86
Average for 8 years........	634	1.74

I was reluctant to discard the race and was tempted to keep it under control, but without such exacting daily observation and recording of the daily division rate. Accordingly, it has been kept in this manner up to the present time (April, 1926). But from time to time thirty-day tests have been made of the vitality of the race under the former rigid culture conditions, and in each case the same general average division rate has been revealed as during the first eight years of its life. For example, during December, 1915, 52 generations; during December, 1917, 56 generations; during November, 1920, 54 generations, and during February, 1926, 53 generations were attained. On the basis of such tests it is fair to make the exceedingly conservative estimate of 600 generations attained each year since May 1, 1915. This would give, in round numbers, 11,700 generations attained by the culture during the 19 years of its life, to date since May 1, 1907. The vitality of the culture is further attested by the fact that it is continuously affording animals for various other experimental studies on *Paramecium* at Yale and elsewhere.



Graph Showing the Rate of Reproduction of the Pedigree Race of *Paramecium aurelia* during the First Eight Years of Culture

The ordinates represent the average daily rate of division of the four lines of the culture, again averaged for each month. The vertical broken lines indicate the limits of the calendar years. The figures 1000, 2000, etc., represent generations and are placed above the periods in which they were attained.

Careful studies of the rate of division of sub-lines derived from the main culture and bred under the most constant environmental conditions revealed the fact that there are inherent, *normal*, minor, periodic accelerations and depressions of the fission rate due to some unknown factor in cell phenomena. These were termed rhythms (5). In a search for the underlying factors of rhythms a complicated internal nuclear reorganization process was discovered and named endomixis (6). This process, in essence, consists of a gradual disintegration and absorption of the macronucleus and the formation of a new one from the micronuclear apparatus. This results in the reorganization of the cell (endomixis) without the coöperation of two animals involving syncaryon formation (amphimixis) as in fertilization.

Endomixis obviously introduces an entirely new problem, though, of course, it in no way affects our former conclusion that fertilization, involving syncaryon formation, is not intrinsically a necessary phenomenon in the life history of the organism. Whether endomixis is a necessary factor for the continuance of the race is another question and is now being intensively investigated (7).

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These two publications contain extended discussions of the problem from different viewpoints.

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EXPERIMENTS ON THE DEVELOPMENT OF THE EAR OF AMBLYSTOMA PUNCTATUM

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THIRTY-ONE FIGURES

CONTENTS

Introduction 13
Normal development 15
Material and methods 17
Experimental 18
  Extirpation of the ear rudiment or of the invaginated ear 18
    Simple extirpation 18
    Extirpation with transplantation of undifferentiated ectoderm 26
  Partial extirpation of the ear at the otic cup and otic vesicle stage 32
    Simple extirpation 32
    Extirpation with transplantation of undifferentiated ectoderm 42
  Reciprocal transplants of half of the ear rudiment between white and pigmented embryos 45
  Transplantation of the entire or partial otic cup into an abnormal location 48
General discussion 50
Summary 53
Literature cited 54

INTRODUCTION

In 1906, a series of experiments on the ear of different species of Amphibia were performed independently by three investigators, Levy and Spemann in Germany and Streeter in this country. An earlier work by Spemann ('02) provided the stimulus for the subsequent research. He found that when a Triton embryo at the neural-fold stage was divided transversely into an anterior and posterior half, each half developed an otic vesicle.[1] It was concluded, therefore, that the cut had gone through those cells which had already been set apart to form the ear.

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[1] Spemann, '02, p. 526.

Using this as a basis for his investigation, Levy ('06) performed certain experiments from which he concluded that the otic cup was a self-differentiating, harmonic equipotential system. In other words, the cells of the auditory cup develop independently of their surroundings and the subsequent fate of the cells with regard to the future labyrinth is not determined. Thus, any half of the otic cup would form a complete labyrinth one-half the size of the normal labyrinth.

Later experiments by Spemann on Rana esculenta embryos did not confirm these conclusions. When the anlage of the ear was divided at the neural-fold stage, both halves did not form complete labyrinths. There was always a consistent absence of the endolymphatic duct in one case. He concluded, therefore, that the open otic cup was not an harmonic equipotential system.

There followed from this a group of experiments involving rotation of the otic cup. Although the results of Streeter and Spemann contradicted each other with respect to the rotation of the vesicle, the two investigators were agreed on one point, namely, that the otic cup was not an harmonic equipotential system, but had already undergone considerable differentiation.

The following experiments on embryos of Amblystoma punctatum indicate quite clearly that the ear rudiment, at the time of invagination to form the otic cup, represents what might be called a transitional stage between a condition of totipotence and one of localization of organ-forming areas. With the closure of the vesicle, differentiation has become established, although a certain amount of reorganization is still possible.

The results show also that a similar condition exists in the surrounding tissue. Coincident with the invagination of the otic cup, the epithelium exhibits a distinct loss in its capacity to regenerate ear tissue.

This work was undertaken at the suggestion of Prof. Ross G. Harrison, and has been carried on under his direction. I wish, at this time, to express my thanks for the suggestions and criticisms which he has offered during the course of the investigation.

NORMAL DEVELOPMENT

The normal development of the ear of Amblystoma has already been fully described by Norris ('92) and Streeter ('21). It may be well, however, for the sake of clearness, to review the more important stages, correlating them with the various stages in the development of the animals as designated by Harrison ('18, '25).

The sensory epithelium first shows indications of thickening at stage 23, shortly after closure of the neural folds. At stage 24 a definite auditory plate is formed which is visible as a distinct white mass of cells lying directly beneath the outer ectodermal layer. Invagination of the auditory plate is evident at stage 25 and the concave pigmented portion—the auditory cup—is visible externally as the so-called 'ear spot' (fig. 3, *AU*). The beginning of closure to form the otic vesicle takes place at stage 27, at which time the dorsal edge of the cup separates from the rest of the sensory epithelium and commences to bend in a ventral direction. Closure is almost complete at stage 29 and fully completed at stage 30 or 31. As a result of this closure, that portion of the otic cup which was originally just above the center comes to lie directly dorsal.

Elongation in a dorsal direction to form the recessus labyrinthi begins at stage 33 and is quite distinct at stage 34. In these stages numerous mitoses are visible, chiefly in the ventral and posterior part. The dorsal part, which forms the recessus, shows no signs of cell division; growth takes place from the cells at the base. It is at stage 34 that muscular contraction is first evident.

A distinct endolymphatic duct is visible at stage 36, and it has taken its position as an extension from the dorsomedian part of the vesicle. The pouches of the anterior and horizontal canals appear at stage 39. These can distinctly be seen to arise from the ventral half of the vesicle. At this stage, also, the vesicle is made up of a single layer of cuboidal cells except for the thicker sensory area on the ventral, and, to a lesser extent, on the posterior wall. The anterior and lateral canals are definitely separated at stage 41, but the posterior canal is not distinct until stage 45 and 46. Up to that time it exists as a dorsal pouch, but the crista acustica is nevertheless present earlier. A definite separation of the sacculus from the utriculus begins at stage 40.

At about stage 39, a single yellow, glistening body is visible externally in the living embryo. It lies within the vesicle near the ventral side and is the beginning of the otolith. This becomes elongated and shows a constriction around the middle, giving the appearance of two smaller otoliths fused together. These separate at stage 40 and one takes a position anterior and slightly lateral to the other. At stage 41–42 a third otolith appears dorsal and median to the other two. All three otoliths increase in size so that they are very distinctly visible from the dorsal side. The anterior otolith lies above the macula utriculi; the posterior otolith, above the macula sacculi; and the median otolith, within the saccus endolymphaticus. The otoliths disappear during fixation. They are probably similar to the structures described by Proebsting ('24) in Triton.

The labyrinth at stage 46 presents in general the appearance shown in the model (figs. 19 and 19 a). The size of the normal labyrinths varied in anteroposterior length in different individuals. The model represents one of average size, although somewhat longer labyrinths were not uncommon. The anterior, lateral, and posterior canals are distinctly formed and the cristae acusticae show as definite sensory areas in the ampullae. The canals open into the utriculus, and the crista of the lateral canal is still connected with the macula utriculi. The sacculus lies ventral and slightly posterior to the utriculus. Three distinct sensory areas may be distinguished, although those of the pars neglecta and lagena are still connected with the macula sacculi. The endolymphatic duct extends between the labyrinth and the medulla. It opens ventrally into the sacculus and is somewhat enlarged above the roof of the medulla to form the saccus endolymphaticus (fig. 12).

In the following discussion certain definite terms will be used to designate particular stages in the development of the ear. Thus, the stage at which the sensory ectoderm becomes thickened (stages 23 and 24) is referred to as the auditory plate. This area, at those stages in which it undergoes invagination (stages 25 to 28), is called the auditory or otic cup. The term auditory vesicle is used to designate the stage at the beginning of closure of the cup (stage 29). Inasmuch as the ear shows quite a distinct difference in its germinal constitution at these different stages, it seems necessary to make a rather arbitrary distinction in the terminology of the discussion.

MATERIAL AND METHODS

The following account is a record of operations made on embryos of Amblystoma punctatum during the years 1923–1925.

The technique of the operations was similar to that used in experiments of this kind (Harrison, '08, '18, '21). The experiments involved removal of the whole or a part of the ear region and, in certain cases, transplantation of tissue from one embryo to another. The individual types of operations will be described in detail in later sections.

In a few cases and where they were abnormal, they were killed at earlier stages. Otherwise the animals developed up to stages 44 to 46. They were then fixed in corrosive acetic, sectioned 8 μ or 10 μ and stained in Delafield's haematoxylin and Congo red or eosin.

EXPERIMENTAL

Extirpation of the ear rudiment or of the invaginated ear

Simple extirpation. These operations involved the removal from the ear region of circular pieces of ectoderm 0.2 mm., 0.3 mm., 0.4 mm., 0.5 mm., and 0.6 mm., respectively. The extirpations were made on embryos from stage 21 to stage 30. The purpose of the experiments was twofold. The removal of pieces of different size was to determine whether regeneration would occur following removal of ectoderm in the ear region and whether such regeneration, if it did occur, would bear any relation to the size of the piece removed. In this way, the extent and relative potency of the ear-forming tissue could be determined. In the second place, operations at different stages in the development of the embryos would determine whether there was any definite relation between the age of the embryo and the amount of regeneration; in other words, whether and to what extent there was a loss in potency with increase in the age of the embryo.

In the first series only that portion of ectoderm was removed which was definitely connected with the formation of the ear. As much of the surrounding ectoderm as possible had to be left intact; but, on the other hand, none of the tissue actually connected with the formation of the ear must remain in the embryo. Consequently, the operation, while fundamentally the same throughout the series, varied slightly in the older and younger stages. In the stages previous to stage 25 there was no external indication of the location of the ear rudiment, and in the earliest stages at which the operations were made there was not even a thickening of the sensory epithelium. Measurement of the thickened ectoderm from cross-sections at stage 23 showed the diameter to be slightly less than 0.2 mm. Therefore, in the stages 21 to 24 a circular piece of ectoderm 0.2 mm. in diameter was removed. This allowed for slight variations in the size of the ear rudiment and at the same time did not remove much of the surrounding tissue. In stages 21 to 23 the position of this piece corresponded with the area shown in figure 1, *AU*.

At first some difficulty was experienced in locating the exact region. It was found, however, that when this particular area was removed the wound healed to a point directly opposite the left ear spot. The left ear spot appeared the day after the operation. If no ear spot was present on the right side, it was considered to be proof of the accurate location of the ear region. At stage 24 the auditory plate was included.

In all cases the size of the cut was measured by means of a micrometer eyepiece. All measurements were made immediately following the cutting of the ectoderm and while the area to be removed was still in position in the embryo. As the incision was made, the ectoderm on both sides of the cut receded somewhat. Thus there was a gaping of the wound which gradually increased until there was a distinct space between the edges of the circular area and that of the surrounding tissue. Measurements made immediately following the operation and before much gaping of the wound had taken place gave less chance of error.

Measurement of the ear at the later stages (stages 25 to 30) showed a variation in the diameter from 0.15 mm. to 0.25 mm. Since the object was to remove all of the tissue directly connected with the formation of the ear, but to leave the surrounding tissue intact, it was necessary to vary the size of piece according to the size of the otic cup. Only that portion of ectoderm immediately overlying the ear was removed.

In the first experiments involving removal of 0.3 mm., 0.4 mm., and 0.5 mm., the method of procedure was a modification of that employed by Spemann ('18) in transplantation experiments on very young embryos. A pipette with a tip measuring the required diameter at its inner margin was placed against the ectoderm in the region of the auditory plate. This ectoderm was drawn a short distance up the tube of the pipette so that it formed a conical projection with a diameter equal to the bore of the tube. A slight depression at the base of the cone marked the line where the cut was to be made. A large mortality resulted from these operations, particularly following removal of 0.5 mm., and was apparently due to the size of the wound. It was thought also that there was possible injury to the brain from the use of the pipette. Consequently, the earlier method was resumed and was more successful.

a. Removal of a piece of ectoderm, 0.2 mm. in diameter (stages 21 to 24). Out of six cases, five practically normal labyrinths resulted. In the sixth case the posterior canal was entirely lacking and the anterior canal ended blindly posteriorly. Four out of the six operations were made at stage 24 when the auditory plate was sufficiently thickened to be visible. Hence, there was no difficulty in locating its position, and, in these cases at least, complete regeneration was not due to inaccurate location of the extirpated area.

Records of size show all of these operated labyrinths to be smaller than the unoperated. The difference, however, was slight (40 μ to 90 μ). This brings out an important fact which was observed not only in this, but in all other series. Wherever complete regeneration occurred, the resulting labyrinth varied only slightly in size and degree of development from that on the opposite side. In some cases, no difference at all could be observed.

Removal of the ear rudiment, particularly in the later stages, must obviously delay, if it does not prevent, the formation of a vesicle. The wound heals in a few hours, but in the majority of cases no vesicle was observed on the day following the operation. Inasmuch as the embryo had reached stages 25 to 27 in that time, the ear spot visible on the left side indicated the invagination of the otic cup. There must have been a difference of at least two days between the development on the two sides of the embryo. On the second day an ear spot was usually visible on the right side, but the relative stage of development in comparison with that on the left side could not be determined. Observation at later stages showed a retardation in the development of the otoliths. But by the time the animals had reached stages 45 and 46 no external differences were visible and, in many cases, the sections showed that the operated ear was practically identical with the unoperated ear in size and development. Evidently there is some acceleration in development which compensates for the delay caused by the operation. This is even more apparent in other series where the stage of operation was later.

b. Removal of the otic cup (stages 25 and 27). These operations produced interesting results. There was no case of complete regeneration. A single case, operated on at stage 27, failed to show any trace of regeneration. The other six cases were divided equally into two groups. The first group showed either partial or complete endolymphatic duct and anterior canal. The posterior canal was present in two cases. The sacculus was very small or absent and the lateral canal, where present, was only a pouch. The second group comprised three cases in which the only regeneration was one or two small vesicles practically lacking in sensory epithelium, but surrounded by a cartilaginous capsule (compare fig. 7).

The operations of this series show clearly that regeneration of the labyrinth follows extirpation of a piece of ectoderm 0.2 mm. in diameter. They show also that there is a very distinct relation between the age of the embryo and the degree of regeneration. Up to stage 24 complete regeneration occurs in the majority of cases. While a vesicle can be formed at stage 25 and later, it is by no means complete. Apparently there is a distinct loss in the regenerative capacity of the surrounding tissue between stages 24 and 25.

c. Removal of a piece of ectoderm 0.3 mm. in diameter. There were only two positive cases among those operated on between stages 21 and 24. Four other cases, in which complete regeneration apparently occurred, showed a vesicle developing on the right side the day following the operation. This brought up the possibility of error in the location of the extirpated area and, consequently, the evidence from these individuals is not considered conclusive.

Of the two positive cases, that in which the operation was performed at stage 21 developed a complete labyrinth. This showed a slight abnormality and reduction in size of the sacculus and contained masses of cells within the lumen. Otherwise it was like that on the left side.

The second case was operated on at stage 23, and the resulting labyrinth showed only a simple vesicle containing sensory epithelium on the medial and ventral walls. The ventral portion of the endolymphatic duct was possibly present.

Considerable reduction in the amount of regeneration was evident when the operation was performed at stage 25. One of the resulting labyrinths lacked the lateral and posterior canals and sacculus, while the second consisted of a small, simple, vesicle containing sensory epithelium.

Still less regeneration followed the three operations at stage 27. A small mass of cells was all that appeared and, in one case, no regeneration whatever took place.

These results show, even more strikingly than the preceding, that there is a progressive decrease in the amount of regeneration with increase in the age of the individual at the time of operation.

d. Removal of a piece of ectoderm 0.4 mm. in diameter. Three complete labyrinths developed following extirpation at stages 20 and 21. The fourth operation performed at this stage resulted in slight abnormalities of the anterior part of the anterior canal and sacculus. The lateral canal was present only in the posterior part. The operation at stage 22 resulted in complete regeneration, but with a general enlargement of the labyrinth.

No endolymphatic duct developed in the single case operated upon at stage 25. A small, simple vesicle with lateral and posterior outpushings regenerated. The anterior crista was possibly present. Even less regeneration took place following extirpation at stage 27. In a second case there was no regeneration, and this was also true in one case operated at stage 30.

These results agree with those of the other two series.

e. Removal of a piece of ectoderm 0.5 mm. in diameter. As shown in figure 1, the extirpated area extended from near the dorsal midline well over the yolk and anteriorly over the mandibular arch. Thus it is unlikely that any operations of this type failed to include the otic plate, since the diameter of the piece removed exceeded that of the plate by 0.15 mm. on all sides. This margin of safety was nearly equivalent to the diameter of the plate itself.

The mortality from this type of operation was high. The wound often opened up again after it had apparently healed. In many cases healing produced a contraction in the head region, drawing the brain down to one side. Since the total number was so small, all the animals were sectioned regardless of the stage at which they were killed.

A single case in which the operation was performed at stage 21 regenerated a complete labyrinth. Two individuals operated at stages 22 and 23 were killed at stages 37 and 36, respectively. The labyrinth in each case had reached the same degree of development as that on the right side and, hence, was classed as a case of complete regeneration. The sacculus was lacking in a second individual operated on at stage 22.

One normal labyrinth followed extirpation of the auditory plate (stage 24), but of the other two at that stage one showed only a small simple vesicle and the other completely failed to regenerate.

A somewhat similar result followed extirpation of 0.5 mm. at stage 25. There was one case of considerable, although incomplete, regeneration and one in which no regeneration occurred. No operations of this type were performed on older stages.

f. Removal of a piece of ectoderm 0.6 mm. in diameter. Since it was evident from the preceding experiments that the limit of the regenerative capacity of the ectoderm in the earlier stages had not been reached, the attempt was made to remove a still larger area.

One operation at stage 22 was successful. The resulting labyrinth, while approximately the same size as the normal, did not show the normal differentiation. It consisted merely in a large simple vesicle with poorly developed sensory epithelium.

Four cases at stage 24 indicated that an area 0.6 mm. in diameter approached the limit within which the ectoderm surrounding the auditory plate might regenerate an ear vesicle. A small mass of cells was all that appeared in two of the cases. The other two completely failed to regenerate.

g. Summary. The results of these experiments show that the ectoderm surrounding the ear region possesses the capacity for regenerating the ear vesicle to a marked degree. The cases of complete failure to regenerate were few, even when the operation was performed at a relatively late stage. Several individuals in which, apparently, no regeneration had occurred were found, upon examination of the sections, to possess at least a small mass of cells.

The extent to which regeneration proceeded varied under different conditions, as can be seen from table 1. It is evident that the results may be divided into two groups, depending on the stage at which the operation was performed. Out of twenty-five cases in which the ectoderm was removed prior to invagination of the otic cup, fourteen showed regeneration of complete labyrinths. On the other hand, there was no case of complete regeneration when the operation was performed at the otic-cup stage. It is evident, then, that the time of invagination of the auditory plate is a period of considerable loss in potency of the surrounding tissue.

The results show, moreover, that in those stages in which no trace of thickened epithelium was present it was not possible to prevent regeneration, even though one operation removed a piece of ectoderm three times the diameter of the normal ear region as it was determined by measurement of the auditory plate. When the auditory plate was included in the extirpated area, it was possible to secure individuals in which no regeneration had occurred. This took place only following removal of 0.6 mm. and, in one case, of 0.5 mm. Other operations of the same type exhibited such slight regeneration that it was considered safe to conclude that 0.6 mm. was the limit beyond which the surrounding ectoderm at the auditory-plate stage did not regenerate an ear vesicle.

After invagination to form the otic cup, it was possible to prevent regeneration by the removal of a considerably smaller piece of ectoderm. The greatest amount of regeneration following removal of 0.3 mm. at stage 27 was a hollow mass consisting of only a few cells, indicating that here also the limit of regenerative capacity had been reached.

TABLE 1

Showing the relation between the amount of regeneration, the size of piece removed, and the age of the embryo at the time of operation

STAGE OF OPERATION	AMOUNT OF REGENERATION	NUMBER OF CASES INVOLVING REMOVAL OF PIECES OF ECTODERM OF DIFFERENT DIAMETERS					TOTAL
		0.2 mm.	0.3 mm.	0.4 mm.	0.5 mm.	0.6 mm.	
20–23	Complete	1	1	4	3		9
	Incomplete	1	1	1	1	1	5
	None						
24	Complete	4			1		5
	Incomplete				1	2	3
	None				1	2	3
25–27	Complete						
	Incomplete	6	4	2	1		13
	None	1	1	1	1		4
	Total	13	7	8	9	5	42

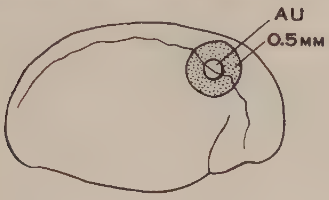
Thus it is quite evident that the ectoderm for at least 0.15 mm. around the normal ear region is capable of regenerating ear tissue. This potency is markedly decreased following invagination of the otic cup. There is not such an evident relation between the amount of regeneration and the size of piece removed except where it approaches the limit of potency.

Extirpation with transplantation of undifferentiated ectoderm. This series was undertaken for the purpose of determining to what extent the presence of undifferentiated ectoderm from another embryo would serve as a check to the regeneration of the vesicle. It had already been found, in working on the limb, that the percentage of regenerating cases was considerably reduced by a similar method and, when a sufficiently large area was involved, regeneration could be completely suppressed (Harrison, '18).

Pieces of ectoderm, respectively 0.2 mm., 0.3 mm., 0.4 mm., and 0.5 mm., were removed from the ear region (fig. 1). Ectoderm of corresponding size from a second embryo which had been stained in Nile-blue sulphate (Detwiler, '18) was transferred to the extirpated area. In twenty-seven cases this graft came from the region just posterior or posterodorsal to the eye (fig. 2, *TR*) of the second embryo. In nine other cases the grafted ectoderm was taken near that area from which the limb subsequently developed. Whenever possible, the reciprocal transplant was made; that is, the ectoderm from the ear region was transferred to that area from which the graft was taken.

This type of operation possessed a distinct advantage over the simple extirpations from two points of view at least. In the first place, both grafts differed in color from the host tissue. The embryos from which the ear region had been removed were unstained and possessed the normal brown color, while the grafted tissue was blue. The reciprocal transplant resulted in a brown graft on a blue embryo. Consequently, the position of each graft could be located up to the stage at which the larva was killed. This was of value particularly in the ear region, inasmuch as the position of the graft could be compared with that of the developing vesicle on the left side. Only those individuals were sectioned in which the records showed that, on the day after the operation, the graft was directly opposite the vesicle on the left side. In the second place, the graft in the stained embryo of ectoderm from the ear region generally developed a vesicle and acted as a further control regarding the accuracy of location of the extirpated region.

a. Removal of ectoderm 0.2 mm. in diameter (stages 21 to 24). Two practically normal labyrinths resulted from operations at stages 21 and 22, respectively. In the first case the graft was taken from the region posterior to the eye and in the second, from the limb region of the stained embryo.



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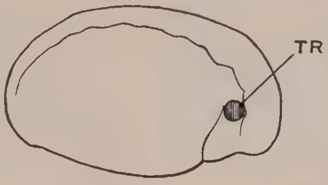

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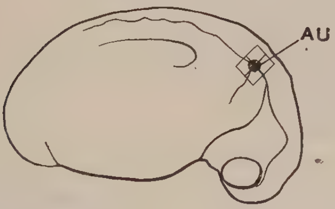

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Fig. 1 Embryo of Amblystoma punctatum at stage 21. × 8. *AU*, ear region; 0.5 mm., size of large extirpation.

Fig. 2 Embryo at the same stage, showing region posterior to eye from which grafts were taken. *TR*, region of transplant.

Fig. 3 Embryo of Amblystoma punctatum at stage 25. × 8. *AU*, ear spot. The square area around the ear spot marks the limits of the areas removed in partial extirpation. (From the plates of normal embryos drawn by Miss L. Krause.)

Of the remaining four cases of this type, operated on at stage 24, one showed complete regeneration. Two other cases showed a reduction in the size of the sacculus and a loss or reduction of one canal. The fourth case is interesting in that the operation took place at a late stage 24. The resulting labyrinth exhibited still further reduction in development, inasmuch as the endolymphatic duct, posterior canal, and sacculus were lacking, and the anterior and lateral canals were incompletely developed (figs. 5 and 6). This corresponds with similar cases following simple extirpation.

b. Removal of the otic cup (stages 25 to 30). These operations also yielded results which were comparable with the results following simple extirpation. Three operated at stage 25 and one each at stages 26, 27, and 28 produced only simple vesicles without endolymphatic duct. The grafts in all these cases came from the region posterior to the eye of stained embryos. One other case at stage 26, in which the graft was taken from the limb region, developed a complete labyrinth. Inasmuch as the reciprocal transplant developed to a certain extent, it is certain that at least a portion of the otic cup was removed.

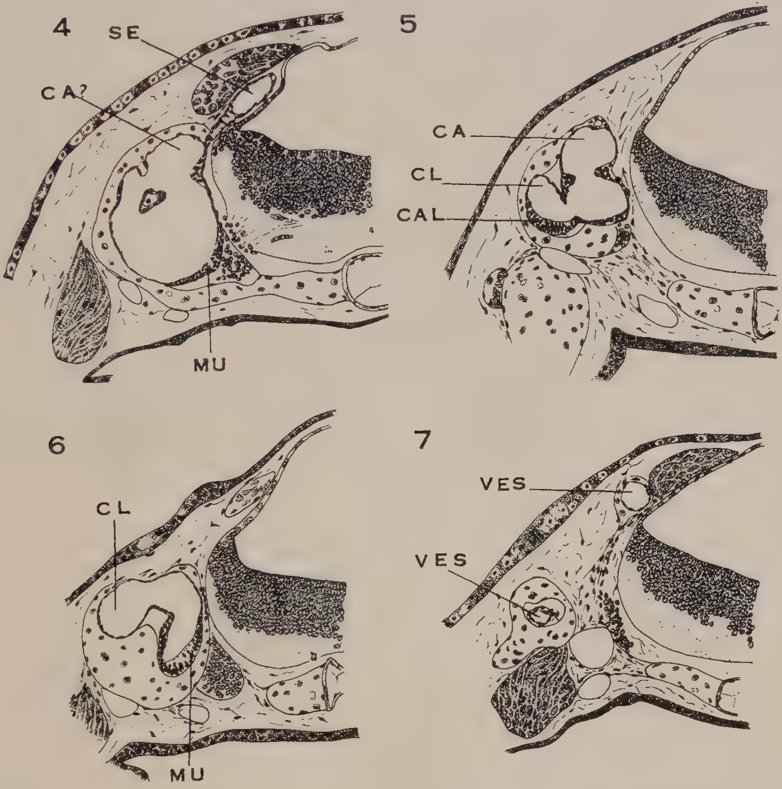
Five experiments were performed on embryos at stage 30, at which time the vesicle had completely closed. In three cases the graft was taken from posterior to the eye and in two, from the limb region. From one of the latter operations a small oval vesicle resulted. Otherwise there was no regeneration.

c. Removal of ectoderm 0.3 mm. in diameter (stages 21 to 27). A single case in which the operation was performed at stage 21 possessed only the endolymphatic and one deep otolith. Study of sections showed that an endolymphatic duct was present, but the remainder consisted of a single vesicle. The suggestion of an anterior canal was due to a transverse bar of cartilage (fig. 4). This is characteristic of another type of operation (compare figure 8).

One individual operated on at stage 23 developed three small disconnected vesicles. An operation at stage 24 produced a single large vesicle without endolymphatic duct and one at stage 27 did not show any trace of regeneration.

d. Removal of ectoderm 0.4 mm. in diameter (stages 21 to 27). Ten individuals were sectioned. A single individual operated on at stage 21 produced a labyrinth lacking anterior and posterior canals, with enlarged endolymphatic duct and small sacculus. A second case was not sectioned, as the location of the graft was doubtful.

Out of four operations at stage 22, two resulted in a simple vesicle without endolymphatic duct and anterior canal. The fourth individual had developed only to stage 37. The regenerated vesicle lacked an endolymphatic duct. It was consid-



Figs. 4 to 7 Sections through labyrinths which regenerated following total extirpation with transplantation of ectoderm. × 50.

Fig. 4 EcT-28. Operated at stage 21. 0.3 mm. removed. Section through anterior part of labyrinth, showing strand of tissue containing cartilage which cuts off a doubtful anterior canal (*CA*). *SE,* endolymphatic sac; *MU,* macula utriculi.

Fig. 5 EcT-36. Operated at late stage 24. 0.2 mm. removed. Section through anterior part of labyrinth. *CL,* lateral canal; *CAL,* crista of lateral canal.

Fig. 6 EcT-36. Section through posterior part of same labyrinth, showing absence of endolymphatic duct and sacculus.

Fig. 7 EcT-63. Operated at stage 23. 0.5 mm. removed. Showing two small vesicles (*VES*) surrounded by cartilage.

erably larger than normal and a differentiation into sensory epithelium had taken place. Two other cases operated on at this stage had grafts taken from the limb region. One of these exhibited considerable regeneration, but the other consisted merely in the saccus endolymphaticus and a small separate vesicle. Two operations at stage 23 resulted in small simple vesicles, one with endolymphatic duct. Complete failure to regenerate followed in one case operated on at stage 27.

e. Removal of ectoderm 0.5 mm. in diameter (stages 22 and 23). Four individuals, two operated on at stage 22 and two at stage 23, were studied. In three cases regeneration consisted in one or more vesicles of small size (fig. 7), one case including also the endolymphatic duct. A fourth case appeared to have a fragmented labyrinth. The various separate portions seemed like parts of a normal labyrinth. The amount of regeneration was considerably greater than in the other two cases and the endolymphatic duct was present. The vesicle began just posterior to the eye, and there may be a possibility that it was not entirely removed and that the part which remained was pushed anteriorly by the graft.

f. Summary and discussion. The results of this series are recorded in table 2. A comparison of this with table 1 shows that the presence of undifferentiated ectoderm does lessen the amount of regeneration. This is most evident when the operation was performed before invagination of the auditory cup. Omitting the operations removing 0.6 mm., for which there is no parallel in the second series, there are nineteen cases involving simple extirpation in the early stages. There were twenty-one cases in which there was a transplantation of undifferentiated ectoderm. A comparison of these two groups shows that the percentage of cases of complete regeneration decreased from 68.4 per cent to 14.3 per cent, while the percentage of cases of incomplete regeneration increased from 26.3 per cent to 85.7 per cent following the transplantation of undifferentiated ectoderm. Moreover, there was a distinct reduction in the degree of development of the regenerated labyrinth in the latter series. The great majority formed only a simple vesicle.

A comparison of operations between stages 25 and 28 with similar cases in table 1 does not show any very striking features. The difference is, again, one of degree of development of the regenerated vesicle. Except for the single case of complete regeneration, which was probably due to an error in the operation, none of the cases in the second series developed more than a simple vesicle. In contrast to this, there were several cases following simple extirpation which showed considerably greater regeneration.

TABLE 2

Showing the effect of transplantation of undifferentiated ectoderm to the extirpated ear region

STAGE OF OPERATION	AMOUNT OF REGENERATION	NUMBER OF OPERATIONS				TOTAL
		0.2 mm.	0.3 mm.	0.4 mm.	0.5 mm.	
20-23	Complete	2				2
	Incomplete		2	8	4	14
	None					
24	Complete	1				1
	Incomplete	3	1			4
	None					
25–28	Complete	1				1
	Incomplete	6				6
	None		1	1		2
	Total	13	4	9	4	30

Observations on the second day after the operation indicated the development of a vesicle either posterior to the graft or in the posterodorsal angle between the circumference of the graft and the dorsal midline of the embryo. This showed conclusively that the vesicle was not formed from the tissue of the graft. In all cases the position of the graft on the day after the operation had been directly opposite the left ear spot.

The consistent appearance in the majority of cases of the regenerated vesicle in a position posterior or posterodorsal to the graft might lead to the conclusion that possibly the extirpation was made from the anterior part of the ear region. Hence, regeneration would be due to that part of the normal ear rudiment which was left in the host. The fact that this occurred in those cases where 0.5 mm. or the otic cup itself was removed would serve as proof against such a conclusion.

A comparison, therefore, of results of extirpations with and without transplantation of undifferentiated ectoderm shows conclusively that the area around the otic plate possesses the power of regeneration of ear tissue to a marked degree. Where no ectoderm is transplanted, there is no decrease in this potentiality for a distance of at least 0.15 mm. beyond the normal ear region, and regeneration is complete in the majority of cases. Where transplantation of ectoderm has been made there is no complete regeneration following extirpation of pieces greater than 0.2 mm. in diameter. Both sets of results show also a considerable reduction in the regenerative capacity of the surrounding tissue with the beginning of invagination of the otic cup.

Partial extirpation of the ear at the otic cup or otic vesicle stage

Simple extirpation. These operations include four series. Experiments were made chiefly on stages 25 and 29 and involved the extirpation of the anterior, posterior, dorsal, and ventral halves of the ear. One half of the otic cup or vesicle, as the case might be, was removed together with the overlying ectoderm. In the majority of cases a small amount of ectoderm on either side of the ear was removed at the same time (fig. 3), and usually that portion of the neural crest lying between the ear and the medullary cord. These precautions were taken in order to make perfectly certain that no cells from the extirpated half remained within the embryo and in order to prevent any possible influence of the ectodermal structures immediately within the extirpated area upon subsequent development. The operations were performed at two definite stages in the development of the ear—the time of invagination of the otic cup (stage 25) and just previous to the complete closure of the vesicle (stage 29).

a. Removal of the anterior half. Out of fourteen embryos operated on at stage 25, nine have been sectioned and studied. One of these had developed up to stage 40 and the others, to stages 44 to 46. The five remaining, judging from their external appearance—that is, number of otoliths—would seem to be similar in their development.

Two of the animals sectioned showed a complete and normal labyrinth. In two cases no canals were present and the sacculus was poorly developed, if it could be said to be present at all. In the other five cases the abnormalities were quite generally distributed over the entire vesicle. The anterior canal in two of these five was constricted off for only a short distance. In two other cases a type of abnormality was present in which a strand of tissue, usually containing cartilage from the capsule, extended across the anterior end from the median to the lateral side (fig. 8). The horizontal canal either remained as a pouch from the utriculus or was only partially separated. The posterior canal was absent in one case out of the five. In all cases the sacculus was smaller than normal, apparently the result of a corresponding enlargement of the utriculus. In all cases the endolymphatic duct was apparently normal. The lagena was present in three cases, but the cristae were generally absent.

Eight of the sixteen embryos resulting from the operation at stage 29 have been studied. The results were similar to those above. There was one case of complete development. Of the remaining seven cases, two did not develop any canals. Three cases showed the characteristic bar of cartilage crossing the anterior part of the labyrinth, and this condition was duplicated in one, across the lateral canal, and in two others, across the posterior canal region. Where this type of abnormality occurred the macula utriculi appeared to extend laterally into the position of the lateral crista acustica. A somewhat similar condition in the posterior part gave the appearance of reduplication, particularly as the sensory area was not well developed and could not be distinguished definitely as belonging either to the macula or crista type. A model of what appeared to be the most pronounced case of reduplication proved to be merely a considerably reduced labyrinth (figs. 20 and 20 a). In six cases the sacculus was either reduced in size or absent, and two cases only showed a normal posterior canal. The type described above, in which the bar of cartilage was present, was not considered normal.

These results indicate that, following extirpation of the anterior half of the ear at the beginning of invagination, a complete labyrinth may develop. This is not generally true at the stage of closure of the vesicle. There is ordinarily a general disturbance in development affecting all parts more or less indiscriminately, but particularly the anterior and lateral canals and, most especially, the cristae and macula utriculi. Operations at stage 29 give results somewhat more uniform than those at stage 25, but, in general, much the same.

b. Removal of the posterior half. Twenty-six embryos in this series survived up to stage 40 or beyond. Of these twenty-six, eighteen embryos were operated on at stage 25 and eight at stage 29.

Ten embryos, operated on at stage 25, were sectioned and studied. One which had developed to stage 40 can only be classed as doubtful, inasmuch as that stage is too early to give positive results as to the degree of development of the various parts of the labyrinth. Two cases showed normal labyrinths, although one was somewhat enlarged throughout. A third labyrinth was normal except for the complete absence of the posterior canal. A simple vesicle only developed in two cases. These vesicles exhibited greater development than did those following extirpation of the entire otic cup. Sensory epithelium of both the macula and crista type was present. In both cases, however, the posterior part of the vesicle was lacking.

The intermediate types exhibited greater uniformity than those of the preceding series. In all cases the posterior canal was lacking and the sacculus was absent or reduced in size (figs. 9, 10, 21, and 21 a). The cristae of both the anterior and lateral canals were generally present, but the canals themselves, particularly the lateral, tended to remain as pouches of the utriculus. In no case did a normal labyrinth result.

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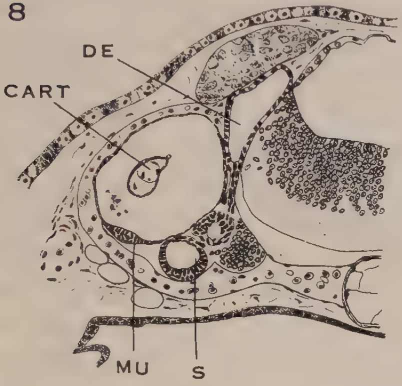

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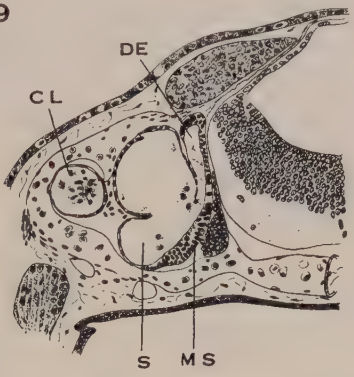

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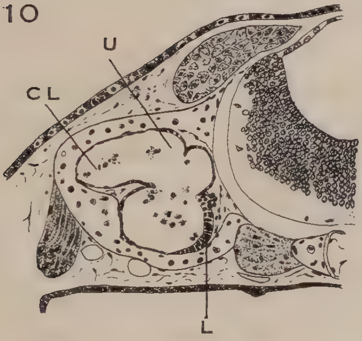

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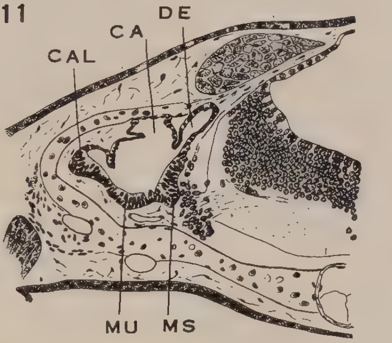


Fig. 8 Ap^{a}-42. Operated at stage 25 +. Killed at stage 45. Showing the characteristic formation of canals in this type of operation. × 50.

Figs. 9 and 10 Ap^{p}-16. Operated at stage 25 +. Killed at stage 45. Sections through the same labyrinth of which the model was made, showing short endolymphatic duct and absence of posterior canal. × 50.

Fig. 11 Ap^{p}-28. Operated at stage 29 —. Killed at stage 44. Showing the reduction in development at a later stage of operation. *CA*, anterior pouch; *MS*, thickening to form rudimentary macula sacculi. Other letters as in preceding figures. × 50.

These results differed from the preceding not only in the general absence of development in the posterior part, but also in the fact that the endolymphatic duct was apparently affected. In two cases the duct was entirely absent and in the remainder, either short—without saccus (fig. 9)—or otherwise abnormal.

The embryos operated on at stage 29 showed certain differences in the nature of the development. One formed a simple vesicle. No posterior canals were present in the other three cases, but there was no case in which the sacculus was entirely absent. The anterior canal was shorter than normal in one case and absent in another. The endolymphatic duct was present in all cases, but in the majority of these it was reduced in size. The horizontal canal, in contrast to the above results, was separated from the utriculus at least for a short distance. Even the labyrinth, which was considered as belonging to the simple vesicle type, showed a certain amount of differentiation (fig. 11). Small pouches were present—probably rudiments of the anterior canal and horizontal ampulla—and considerable development of sensory epithelium. The posterior part was lacking.

It would appear, then, that normal development may follow extirpation of the posterior half of the ear at the otic-cup stage, but not at the otic-vesicle stage. The majority of cases show abnormalities which are more localized than those following removal of the anterior half. There is a consistent absence of development in the posterior half of the vesicle. The endolymphatic duct and sacculus are absent or reduced in size. The horizontal canal is less defective following extirpation at stage 29.

c. Removal of the dorsal half. Twenty-four individuals survived this operation. Of this number, thirteen were operated on at stage 25 and eleven at stage 29.

Out of nine embryos operated on at stage 25, the ears in three were normal. A fourth was normal except for the absence of the saccus endolymphaticus. The five remaining lacked the endolymphatic duct. One case formed a single large vesicle with lateral and ventral pouches. The anterior canal was normal in three of the other cases and somewhat reduced in size in the fourth. The lateral canal showed a tendency toward incomplete separation from the utriculus, but not to the extent that was manifest in the series involving removal of the posterior half. The cristae were generally present. The sacculus was either absent or poorly developed.

Eight individuals operated upon at stage 29 were sectioned and studied. These exhibited considerable uniformity in the nature of the development. In not a single case was the endolymphatic duct present. Six formed single vesicles, of which five were more or less enlarged (fig. 12), and the sixth consisted of two small separate vesicles. The swollen vesicles were irregular in outline or showed pouches which could be identified as the canals or sacculus (fig. 14), and in some cristae were present (fig. 13). A lateral canal was present in the two remaining cases, one posterior canal and no anterior canals. The sacculus was present, but abnormal. In general, the sensory epithelium was poorly developed (fig. 12) or absent altogether.

There is a distinct difference between the results in operations performed at stages 25 and 29. In the earlier stage complete development is possible. Extirpations, while distinctly affecting the endolymphatic duct, did not so seriously affect other parts of the labyrinth. There was a greater number of normal labyrinths from this series than from any of the others. On the other hand, when operations were made at stage 29, the characteristic result was a large, swollen vesicle with one or more pouches and complete absence of the endolymphatic duct.

d. Removal of the ventral half. Out of the twenty-nine individuals which survived up to stage 40 or beyond, eighteen were operated on at stage 25 and eleven, at stage 29.

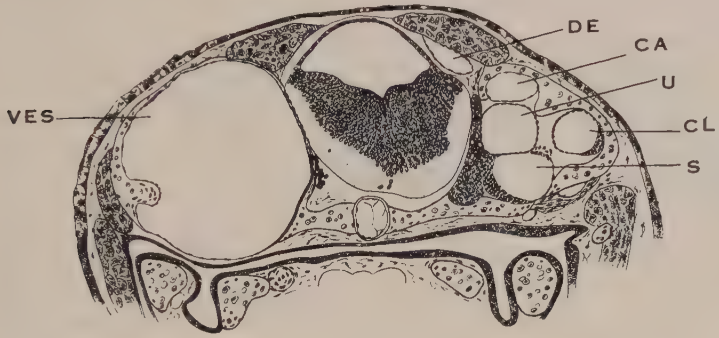
One case of complete development was observed among eight individuals operated on at stage 25. In all eight cases the endolymphatic duct was present, although in one case the saccus was lacking. Similarly, the anterior canal was present, but with the ampulla lacking in one case. The lateral canal was absent in one case and existed as a lateral pouch in four cases. One individual lacked the posterior canal. The sacculus was, for the most part, not differentiated from the utriculus and was not well-developed in those cases where it was distinguishable. In general, development was more complete than in the other types in that all parts of the labyrinth were present. The model (figs. 22 and 22 a) shows the type of development characteristic of this series. Figures 15 and 16 represent sections through this labyrinth.

Results of operations made at stage 29 show, in the majority of cases, less development than in operations made at stage 25. There is no case of normal development. One labyrinth approached the normal, but was considerably smaller and showed a reduction in the sacculus and anterior ampulla. A second labyrinth showed a normal horizontal canal and sacculus, but no anterior canal. The three remaining formed simple vesicles with distinct sensory epithelium (fig. 17). The endolymphatic duct was present and practically normal in all cases.

A complete labyrinth is possible from this type of operation also. The endolymphatic duct is apparently unaffected. Except for consistent loss or reduction of the sacculus and the formation of a lateral pouch, abnormalities resulting from operations at stage 25 are less extreme than in the other types of operations. The characteristic type of development following operations at stage 29 consists in a more or less pear-shaped vesicle at the end of the endolymphatic duct. The walls are of columnar cells somewhat like those of the macula sacculi.

e. Summary and discussion. Comparing the results of the operations performed at stage 25, it was found that normal development resulted in some cases following extirpation of each of the halves. A total of all four types of operation showed normal development in 25.7 per cent of the cases, but the proportion varied with each type. Thus, two normal labyrinths resulted out of nine operations involving the anterior or posterior half, while removal of the dorsal half resulted in four normal out of nine cases and, of the ventral half, in one normal out of eight cases.

The records show, also, that defects and abnormalities were quite generally distributed throughout the resulting labyrinth and, ordinarily, more than one part was affected. There was, however, a characteristic defect or abnormality for each type of operation.



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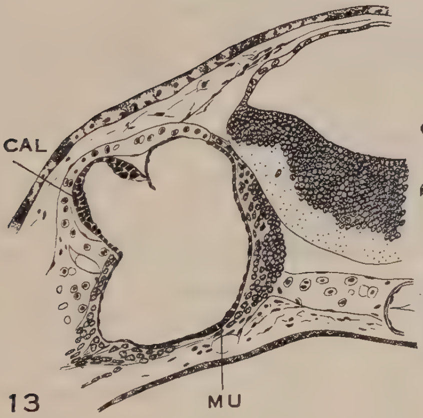

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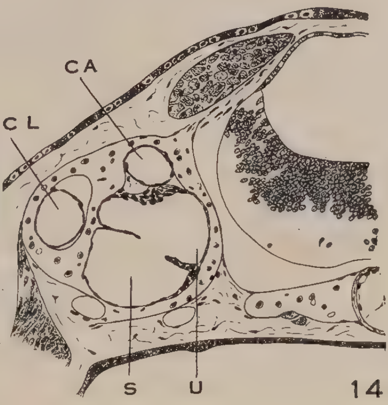

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Figs. 12 to 14 Sections showing development of labyrinth following removal of the dorsal half of the otic vesicle.

Fig. 12 Ap^d-12. Operated at stage 29. Killed at stage 46. Section showing typical swollen vesicle which has enlarged until it presses on the medulla and almost closes the pharynx. Compare its size with that of the normal left labyrinth. × 26.

Fig. 13 Ap^d-45. Operated at stage 29. Killed at stage 46 +. Showing lateral pouch with fairly well-developed crista, also showing macula(?) made up of cuboidal cells. × 50.

Fig. 14 Ap^d-46. Operated at stage 29. Killed at stage 46 +. *CL*, separate vesicle in position of lateral canal. *CA*, separate vesicle in position of anterior canal; *S* and *U*, parts of third vesicle, suggesting sacculus and utriculus. × 50.

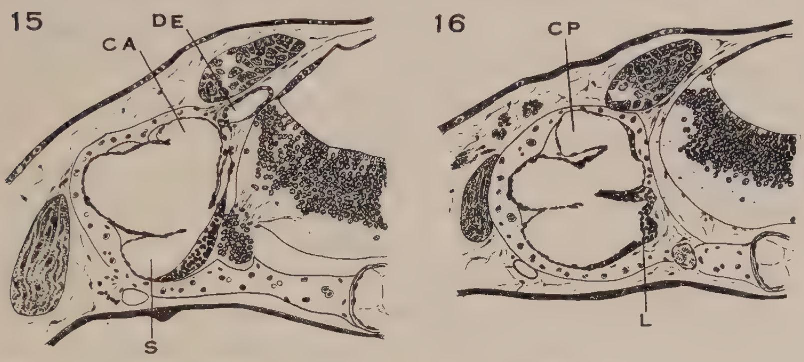
The number of cases of complete development resulting from partial extirpations, together with the generalized nature of the majority of the defects in the abnormal labyrinths, would argue for the totipotence of the ear at this stage. The complete labyrinth of half size which Levy expected did not occur, however. Whenever a complete labyrinth resulted, it was the same size as the normal. Even the incomplete labyrinths were in most cases larger than half normal in size. This is evident from a comparison of the reconstructions (pl. 1). The only cases which approach the half size are those in which very little development took place.

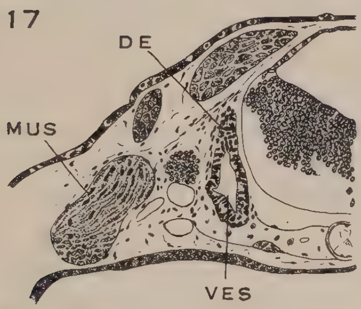
That a certain amount of differentiation has already begun, however, seems to be evident from the results. In more than half of the cases following extirpation of the dorsal half of the auditory cup there is a failure of the endolymphatic duct to develop. Moreover, in one of the so-called cases of complete development, the duct is reduced in length.

Again, following extirpation of the posterior half of the otic cup, there is a distinct absence of the posterior half of the labyrinth, but this may possibly have a slightly different explanation. Observation of normal development of the ear disclosed the fact that apparently the greatest number of mitoses were located in the posterior and ventral parts of the vesicle. There was a conspicuous absence of cell division, particularly in the anterior part. The cells which contributed most extensively to the growth of the vesicle came, then, from the posterior half of the otic cup. Removal of these cells would of necessity produce a marked effect on subsequent development. In a certain sense, therefore, it can be said that at stage 25 there is also localization in the posterior half of the ear. This would account for the characteristic reduction in size of the endolymphatic duct inasmuch as elongation of the recessus apparently takes place from the cells at its base.

Streeter ('07) found similar cases of incomplete development in his experiments involving transplantation of the entire vesicle. He found that the defects might involve any part of the labyrinth and that they varied greatly in their extent. He considered that "such localization of abnormal development is evidence of the high degree of specialization of the cells forming the primitive ear vesicle."[2]

A further tendency toward localization into definite organ-forming regions is substantiated by the experiments on the ear at stage 29. There were only two cases of complete development from these operations. The results point still more conclusively toward the existence of a definite area which will give rise to the endolymphatic duct. In no case, as has already been stated, did an endolymphatic duct develop following extirpation of the dorsal half of the vesicle.





Figs. 15 and 16 Ap^{v}-19. Operated at stage 25 +. Killed at stage 44 +. Sections through anterior and posterior part of labyrinth of which the model was made.

Fig. 17 Ap^{v}-28. Operated at stage 29. Killed at stage 44 +. Showing the typical single vesicle at the end of the endolymphatic duct. *MUS*, muscle.

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[2] Streeter, '07, p. 435.

Removal of the posterior half resulted in the consistent absence of the posterior canal and the absence or reduction in size of the sacculus.

The results of the experiments at the two stages taken in connection with the observations of normal development lead to the conclusion that definite organ-forming areas can be located in the otic-vesicle stage with a fair degree of accuracy. The variations in the results of operations at stage 25, where some cases point toward equipotentiality and others toward localization, indicate that this is the transitional stage. The change from totipotence to localization is evidently a gradual one, and stage 25 marks the end of the one and the beginning of the other. At this stage the only region which can be determined with any degree of certainty is that which goes to form the endolymphatic duct. This agrees with the results of Eisinger and Sternberg ('24), who state that the part of the vesicle from which the ductus and saccus endolymphaticus are formed is determined very early.

Extirpation with transplantation of undifferentiated ectoderm. These series involve removal of the anterior, posterior, dorsal, and ventral half of the otic cup, respectively. Into the extirpated area was transplanted undifferentiated ectoderm from another embryo. Whenever possible, as in previous experiments, the reciprocal transplant was made.

The series were undertaken in an attempt to determine to what extent the presence of undifferentiated ectoderm would serve as a check to development. There was a possibility, in the preceding experiments, that the ectoderm of the surrounding region had taken part in the development of a complete labyrinth following extirpation at the otic-cup stage. The presence of undifferentiated ectoderm from another embryo would prevent this area from healing in to a position where it could become incorporated with the ear tissue to form the resulting labyrinth. Hence, development would proceed from the ear alone.

Approximately half of the grafts came from the limb region and the remainder, from posterior to the eye of the stained embryo. As in the cases of total extirpations, the location from which the graft was taken apparently had no effect on the results. In the majority of cases the reciprocal transplant was made and developed to such an extent that it was recognizable as ear tissue. Thus, it was evident that at least a portion of the ear had been removed by the operation.

a. Removal of the anterior half. Nine individuals of this series were sectioned and studied, including also in eight cases the stained individuals in which the transplanted half developed. The operations involved a series of stages from the time the sensory epithelium had thickened to form the auditory plate until a well-formed otic cup was present (stages 24 to 27).

In all respects the results were similar to the cases of simple extirpation. Complete labyrinths formed in certain cases and the remainder showed the characteristic abnormalities of the anterior canal and absence of cristae.

b. Removal of the posterior half. In this series also, nine cases were studied. Eight of the reciprocal transplants showed a developing vesicle. The operations included stages 24 to 28.

The results, except for three cases of complete development, were quite uniform and were similar to the corresponding simple extirpations. There was a consistent absence of the posterior canal, abnormality of the endolymphatic duct, and absence or abnormality of the sacculus.

c. Removal of the dorsal half. The five cases—three at stage 25, one at stage 26, and one at stage 27—all resulted in the typical enlarged, simple vesicle lacking an endolymphatic duct. These are like the labyrinths which developed from simple extirpations at stage 29.

d. Removal of the ventral half. The operations were performed on slightly later stages than in the first two series. They comprised stages 25 to 29. In eight cases the transplanted vesicle was present in the stained embryo.

The five cases in which the grafted ectoderm was taken from the limb region developed the characteristic pear-shaped vesicle of sensory epithelium at the base of the endolymphatic duct. This was true also in two cases where the graft came from posterior to the eye. Two other cases developed normal labyrinths; complete absence of any vesicle occurred in one case, and in three cases there was some development of canals and sacculus. In all cases the endolymphatic duct was present.

e. Summary and discussion. The results of these series showed conclusively that the presence of undifferentiated ectoderm from another embryo served in no way as a check to the developing vesicle. In all four series the labyrinths were strikingly similar to those in the corresponding series of simple extirpations (table 3). If anything, the results in this series were more uniform in showing the defects characteristic of each type of operation.

The number of complete labyrinths following from all operations was nine—exactly 25 per cent of the total number of cases studied. Here, again, the results are comparable to those of the simple extirpations.

In consideration of the large area of epithelium which was capable of regenerating ear tissue, there was the possibility that grafts from posterior to the eye might possess that potency and might take part in the development of the operated vesicle. This objection was answered by other experiments in which the grafted ectoderm came from the limb region and, hence, could not have the slightest connection with the formation of ear tissue. Moreover, if such an incorporation of the superficial ectoderm did take place, either through its own potency or through the organizing influence of the developing vesicle, there should have been a greater number of cases with normal labyrinths.

The results of these experiments, then, substantiate those of the preceding. In addition, the presence of vesicles developing from the reciprocal transplants served as controls which were lacking in the simple extirpations. Thus, the same conclusion can be drawn which has previously been stated. In the course of its development, the ear proceeds from a condition of totipotence to one of definite localization of organ-forming areas. At the time when invagination occurs and throughout that period in which the otic cup is forming, the ear passes through a transitional stage. During this time there is a gradual increase in the organization of the ear tissue and a consequent decline in the power of any half to develop a complete labyrinth.

TABLE 3

Showing a comparison of results following partial extirpation with and without transplantation of undifferentiated ectoderm

PART REMOVED	TYPE OF OPERATION	TOTAL NUMBER OF OPERATIONS	NUMBER OF CASES EXHIBITING DIFFERENT DEGREES OF DEVELOPMENT				
			Complete	Incomplete			
			All parts present	Organs present, but abnormal	One or more parts entirely or partly absent	Simple vesicle	None
Anterior	Simple extirpation	17	3	4	6	4	
	With transplanted ectoderm	9	4	4		1	
Posterior	Simple extirpation	13	2		8	2	
	With transplanted ectoderm	9	3	2	4		
Dorsal	Simple extirpation	17	4		6	7	
	With transplanted ectoderm	5				5	
Ventral	Simple extirpation	13	2	5	3	3	
	With transplanted ectoderm	13	2	1	2	7	1

Reciprocal transplants of half of the ear rudiment between white and pigmented embryos

In view of the results of the previous experiments, it became of great interest to determine the prospective significance of the auditory plate. If the fate of the different quadrants of the ear rudiment were known in normal development, it might offer an explanation for the abnormalities following partial extirpation. Such experiments had been performed quite satisfactorily on the limb rudiment (Swett, '23). However, staining with Nile-blue sulphate, which was the method used in the case of the limb, is not at all feasible for similar experiments on the ear. Accurate observations of the development of the labyrinth can be made only from sectioned material, and Nile-blue sulphate disappears during the process of fixation.

The finding of a bunch of pure white Amblystoma eggs in the spring of 1925 made the desired series of experiments possible. Reciprocal transplants were made between the white and the normal brown embryos.[3] This was far superior to the use of the vital stain inasmuch as there was a great contrast between the pigmented and unpigmented tissue (figs. 27 and 28). This contrast was visible after sectioning and staining. Hence, it was possible to obtain a very clear idea to what extent the different quadrants of the auditory plate entered into the formation of the developing vesicle.

The embryos were operated on, for the most part, at stages just previous to and at the time of formation of the auditory plate. Five had proceeded as far as the beginning of invagination.

Two types of operations were made. Ten operations involved reciprocal transplantation of the ventral half of the ear region and an equal number involved the anterior half. Thus, in the white embryos, the dorsal and posterior half-vesicles were from the host and the ventral and anterior halves from the pigmented embryo. In the normal brown embryo the reverse condition existed. Since two animals—a white and a pigmented—were used for each operation, a total of forty individuals was obtained, of which twenty-five were sectioned and studied.

The results were strikingly uniform. Although, at the time of operation, no trace of the thickened epithelium was present in a number of cases, later observations showed that the operation had divided the ear rudiment exactly in half (fig. 18). As development proceeded, the superficial ectoderm over the anterior half of the vesicle invariably moved still further anteriorly, while that over the ventral half remained in its original position (figs. 27 and 28). These figures show also that the extent of the pigmented and unpigmented portions of the vesicle could be seen in the living embryo.

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[3] Transplantation experiments between two species of Rana exhibiting differences in embryonic and adult pigmentation were performed by Harrison in 1898. Spemann, also ('18), used this method successfully between different colored eggs from the same species of Triton.

A study of normal pigmented embryos showed that the pigment characteristic of the early stages of development tended to disappear with the thinning out of the epithelium of the vesicle. At the time at which the embryos were killed (stages 36 to 38) the pouches for the anterior and lateral canals were distinct, the endolymphatic duct occupied its final position on the median side of the vesicle, and sensory epithelium had developed in a continuous band extending anteriorly and laterally from the median and ventral wall. Thus, sufficient development had taken place to determine the future divisions of the labyrinth, and the pigment was still visible.

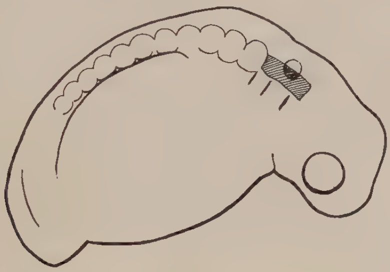

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Fig. 18 TrAl-dv 4a. Camera drawing of embryo four days after operation. The shaded area shows the size and position of the graft from the pigmented embryo. It can be seen that the auditory cup has been exactly divided in half by the operation. The three lines extending below the graft are due to pigmented mesectoderm cells which have migrated over the visceral arches from their original position in the graft. × 12.

The results from the transplantation of the ventral half of the rudiment were almost identical (figs. 24, 24 a, 29, and 30). From the dorsal half arose only the endolymphatic duct and a fan-shaped area on the median wall, representing the macula sacculi. The remainder of the vesicle, including the canals, utriculus, and the lateral wall of the sacculus developed entirely from the ventral half. Laterally, the line of division between the pigmented and unpigmented portions came either exactly at the base of the endolymphatic duct (fig. 29) or a few cells lateral to it (fig. 30). This point, then, marks the meeting-place between the dorsal and ventral halves; in other words, the point of closure of the vesicle.

The results in the other group of experiments were likewise uniform except for slight irregularities. This operation was more difficult than the preceding, and it is possible that the line of incision ran obliquely through the ear rudiment instead of exactly dorsoventral. It is certain that the final line of separation between the pigmented and unpigmented portions was consistently oblique in an anterodorsal to posteroventral direction (figs. 25 and 26).

It is equally possible, however, that such a shifting from the original position takes place normally in development and may be correlated with the greater amount of cell division in the posteroventral portion, as previously observed. This would offer an explanation for the development of an anterior canal following extirpation of the anterior half of the otic cup and the somewhat unexpected abnormalities in the other portions of the labyrinth. The results, however, show definitely that the anterior and lateral cristae and macula utriculi come from the anteroventral quadrant of the vesicle and that the anterior half apparently takes very little part in the formation of the endolymphatic duct.

There is thus a very definite correlation between these results and those following partial extirpation. It is evident that the prospective significance of the various quadrants determines in a large measure the type of labyrinth which develops following removal of any particular part of the otic cup.

Transplantation of the entire or partial otic cup into an abnormal location

Several points of interest arise in connection with the graft in abnormal location in the stained embryo. These seem worthy of brief mention. Out of the seventy-five cases involving transplantation, forty-four were successful in that a vesicle developed. The remaining individuals either died or showed no trace of a transplanted vesicle.

It is evident, then, that in all these cases two auditory vesicles developed from the tissue which normally would give rise to one. Either the regeneration of a second vesicle took place after the extirpation of the entire rudiment or a vesicle developed from each half of the same ear.

All the vesicles which developed in the transplants exhibited practically the same degree of differentiation. This was true whether the graft came originally from the ear region before the formation of the otic cup, whether it included the entire otic cup or vesicle, or whether only half of the otic cup was present. They can thus be considered collectively.

Considering first those transplants to the region posterior to the eye, by far the greater number developed into structures similar to those described by Streeter ('06). Four vesicles developed to such an extent that they suggested the definite parts of a labyrinth, but the tendency was to form single vesicles which conformed more or less to the space between the brain, eye, and epidermis. In a few cases the vesicles developed partly within the eye or brain and in one case, within the gasserian ganglion. Sensory epithelium was present in eighteen out of the twenty-eight vesicles. In some cases this could be definitely distinguished as belonging to the crista or macula type, but in others it was confined to rather doubtful-appearing cuboidal cells. The vesicle which showed the greatest development was one which was transplanted at stage 30, the time of closure of the vesicle. This showed an endolymphatic duct, anterior and lateral pouches, and saccular pouch. The maculae also were well developed.

Those vesicles transplanted to the limb region showed somewhat less development. The majority of these were half-vesicles, but they corresponded to similar half-vesicles transplanted to the head region. The remainder were entire vesicles at stages 26 to 30. They were located, for the most part, within the body wall ventral to the articulation of the humerus with the scapula and lateral to the liver. In some cases the vesicle protruded into the liver and, in one case, it extended into the pericardial cavity. In none of these vesicles was there anything but slight development of sensory epithelium; and in no case was there a trace of a cartilaginous capsule, even though the vesicle lay near the pectoral girdle.

A point which seems worthy of mention was the relative development of these vesicles as compared with those in the embryos from which they originally came. The vesicle which developed in the normal location, whether regenerated from the surrounding tissue or developed from one half of the already formed otic cup, showed greater differentiation generally than that which developed in the abnormal location. In the cases of partial extirpation, the two halves were of approximately equal potentiality. Yet, in different environments they developed differently. This cannot be due to lack of space for development, as was suggested by Sternberg ('24) as the explanation of his results. It would indicate that the ear is not strictly self-differentiating in the sense used by Levy ('06). It would approach more nearly his idea of 'mixed differentiation,' which involves a combination of self-differentiation and that resulting from the action of the environment. This condition is more accurately described by Streeter when he says:

> Transplantation of the ear vesicle shows that the group of cells forming the auditory cup . . . . is specialized to that degree that although removed from their natural relations and placed in a new environment they still continue to differentiate . . . . into a structure approximating the normal labyrinth.[4]

GENERAL DISCUSSION

The main points of the paper have already been discussed as they arose in connection with the different types of operations. Consequently, only a brief review is necessary to bring out their relation to some of the important features of embryological development. It has been found in the case of the limb (Harrison, '18) and more recently of the balancer (Harrison, '25) that the areas from which these organs develop are determined at a very early period in the life-history of the embryo. There are not, in the first stages, definite limits to those areas within which the different organs are formed. The surrounding tissue was found to have the potency of organ formation with a gradually diminishing intensity at greater distances from the center of the organ-forming region. This condition was brought out by the fact that organs regenerated following extirpation of tissue from particular areas. It was found, moreover, in the case of the balancer at least, that the tissue just previous to and at the time of visible differentiation behaved in a more specific way than tissue taken at earlier stages.[5] In a recent paper ('25) Tokura describes a similar condition in Bufo japonicus. He removed a large area of epithelium from embryos with open and with closed medullary folds. While regeneration of the ear proceeded following both types of operation, there was a lessening of potency at the later stage.[6]

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[4] Streeter, '06, p. 558.

The experiments involving extirpation in the ear region confirm the opinion that the ear resembles the other organs in this respect. It was found that the tissue surrounding the developing ear possesses the potency for ear formation to a marked degree. Moreover, there was observed a distinct relation between the amount of regeneration and the age of the embryos at the time of operation. Previous to invagination of the sensory ectoderm to form the auditory cup, regeneration is generally complete following extirpation of areas up to 0.5 mm. in diameter. A distinct loss in the amount of regeneration is evident with the beginning of invagination and the distinct differentiation of the sensory ectoderm.

The greater part of the problem deals with the potencies of the ear itself. Previous work on the ear at the time when invagination had begun had led to varying conclusions, since the results of the experiments seemed conflicting. It was not definitely determined whether the ear at this stage was a self-differentiating equipotential system or one that was highly specialized. The conclusion toward which the results of the present investigation lead is that the ear, at the time of formation of the otic cup, is not in a strict sense a self-differentiating equipotential system.

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[5] Harrison, '25; p. 411.

[6] Tokura, '25, p. 191.

It is self-differentiating in that its development is not determined in the first place by the development or presence of any other organ. The amount of differentiation of the labyrinth is, however, dependent to a considerable extent on the position of the vesicle with relation to other organs. In other words, the auditory cup is capable of forming a distinct vesicle and of initiating the first steps in the differentiation of the labyrinth, but the formation of a normal organ or one which approaches the normal is dependent on its relation to the surrounding structures.

A similar statement can be made with regard to equipotentiality. One of the most important criteria in connection with the totipotence of an organ is the development of a complete whole from any half. A study of the question from this point of view has been the main object of this investigation. The results at first seemed conflicting. Approximately 25 per cent of the cases produced normal labyrinths, indicating that the otic cup was equipotential. In the other 75 per cent the defects which occurred, although inclined to be generalized in their distribution, nevertheless indicated the beginning of a definite localization into organ-forming areas. Similar operations on embryos in which the auditory vesicle was just formed indicated that localization was then more definitely established. The explanation of the apparently conflicting results seems to be that the auditory cup constitutes a transitional stage between a condition of equipotentiality and one in which the various organ-forming areas are distinctly localized.

Thus, it is seen that, in the case of the ear at least, the processes involved in the development of the individual organ parallel those of the embryo as a whole. Although the potencies with regard to each organ or part of an organ are present at a relatively early stage of development, nevertheless other cells which normally would have quite a different fate may, upon the proper stimulus, enter into the formation of the part in question. As the age of the embryo increases, the differentiation of these organ-forming areas becomes increasingly greater until, at about the time when they can be observed as distinct entities, there is a definite localization of tissue which cannot be altered.

SUMMARY

Results of the investigation based on the animals studied:

1. The ectoderm for a considerable area around the normal ear region is capable of regenerating an ear vesicle.
2. There is a very definite relation between the amount of regeneration and the stage at which the operation is performed. A distinct loss in potency occurs after invagination of the otic cup.
3. The amount of regeneration is also dependent to a certain extent on the size of the piece removed.
4. Regeneration is possible following extirpation of the ear region and the transplantation into this area of undifferentiated ectoderm from another embryo.
5. Transplantation of undifferentiated ectoderm from another embryo into the extirpated area lessens the amount of regeneration.
6. The relation between the amount of regeneration, the age of the embryo at the time of operation, and the size of the piece transplanted is similar to that in the case of simple extirpation.
7. Complete development follows in approximately 25 per cent of the cases after removal of half of the ear at the time of invagination. This is not true after closure of the vesicle.
8. In 75 per cent of the cases following partial extirpation abnormal labyrinths result. These show a tendency toward characteristic defects depending on the half which has been removed.

9. The presence of undifferentiated ectoderm in the extirpated area does not check development following partial extirpation. The resulting labyrinths are similar to those in which no ectoderm was transplanted.

10. Reciprocal transplants of one-half of the ear region between white and pigmented embryos indicate that the endolymphatic duct and macula sacculi develop from the dorsal half of the otic cup; the remainder of the vesicle comes from the ventral half; the anterior and lateral cristae and macula utriculi, from the anteroventral quadrant.

11. The two halves of the auditory cup are capable of development independently into more or less complete labyrinths.

12. That half which was transplanted into an abnormal location underwent much less development, in general, than the half which remained in its normal location.

13. From the above results it is concluded that the ear at the otic-cup stage represents a transitional stage between a condition of totipotence and one of equipotentiality.

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ABBREVIATIONS

SE, endolymphatic sac
DE, endolymphatic duct
CA, anterior canal
CP, posterior canal
CL, lateral canal
S, sacculus
L, lagena
CAA, crista of anterior canal
CAP, crista of posterior canal
CAL, crista of lateral canal
MU, macula utriculi
MS, macula sacculi

PLATE 1

EXPLANATION OF FIGURES

19 to 22 Reconstructions of labyrinths made by the wax-plate method. Lateral view. In all figures the anterior part is at the left. Sensory areas are painted gray. × 50.

19 a to 22 a Median view of same reconstructions. × 50.

19 and 19 a Normal labyrinth. Stage 46.

20 and 20 a Typical labyrinth following removal of anterior half of ear at stage 29. The canals were formed by a transverse bar of cartilage. Stage 45 (Ap^{a}-45).

21 and 21 a Showing complete absence of the posterior half of the labyrinth following removal of the posterior half of the ear at stage 25 +. Stage 45 (Ap^{p}-16).

22 and 22 a Showing characteristic lateral pouch (*CL*) following removal of ventral half of ear at stage 25 +. Stage 44 + (Ap^{v}-19).

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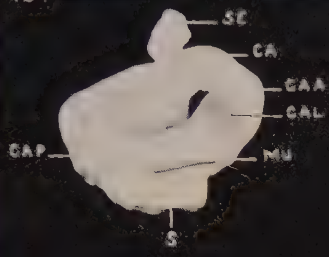

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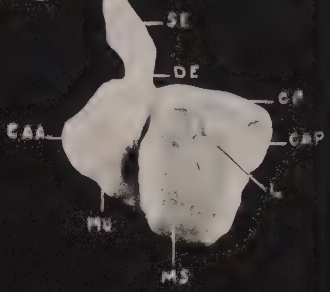

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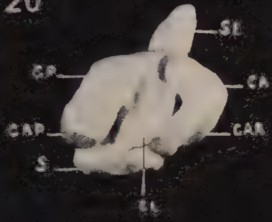

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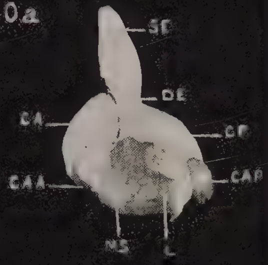

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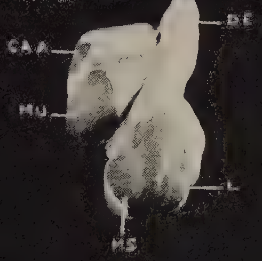

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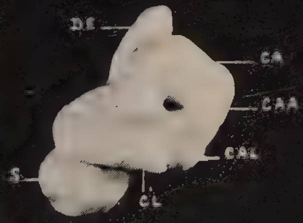

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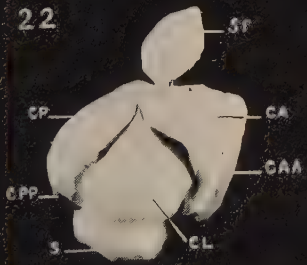

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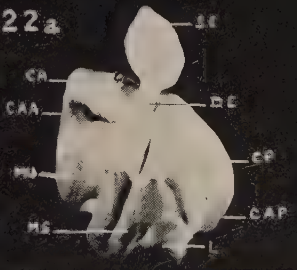


PLATE 2

EXPLANATION OF FIGURES

Reconstructions of labyrinths formed of pigmented and unpigmented tissue. White areas represent the portions of labyrinths formed from unpigmented tissue. Gray areas represent the portions formed from pigmented tissue. × 100.

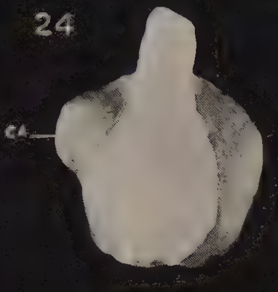
24 Median view, showing the prospective significance of the dorsal and ventral halves of the ear rudiment. The endolymphatic duct and macula sacculi are formed from the dorsal half (host) and the remainder of the vesicle, from the ventral half (graft). (TrAl-dv 4a.) Compare figure 27.

24 a Anterior view of the same labyrinth.

25 and 26 Lateral view of two labyrinths in which the anterior halves were interchanged at stage 24. These show the ventral shifting of material of the anterior half and the formation of the endolymphatic duct almost entirely from the posterior half. Compare with figures 20 and 21. (TrAl-ap 2a and 2 p.)

25a and 26a Median views of the same labyrinths.

24



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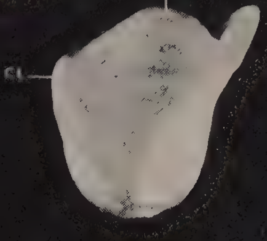

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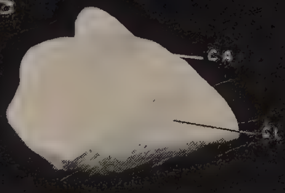

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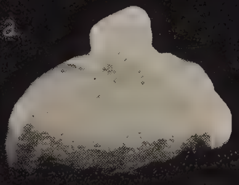

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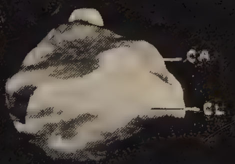

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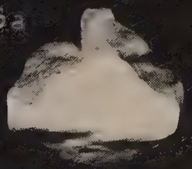


PLATE 3

EXPLANATION OF FIGURES

27 Camera drawing of living animal seven days after operation in which the ventral half of the ear rudiment was grafted from a pigmented embryo. The pigmented portion of the developing vesicle shows as a dark circle, and above it lies the grafted epidermis. Same individual as figure 18 (TrAl-dv 4a). × 18.

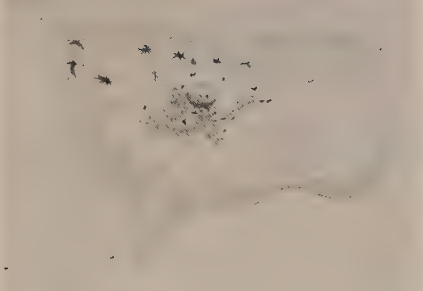
28 Drawing of living animal six days after operation. This shows the pigmented anterior half of the vesicle, also the characteristic migration of the epidermis in this region (TrAl-ap 8a). × 18.

29 Section through the labyrinth of a white embryo in which the ventral half of the ear rudiment came from a pigmented embryo. This shows the sharp separation of pigmented and unpigmented tissue at the base of the endolymphatic duct (TrAl-dv 1a). × 100.

30 Section through the labyrinth of a pigmented embryo in which the ventral half of the ear rudiment came from a white embryo. This shows the same sharp separation of pigmented and unpigmented tissue at the base of the endolymphatic duct (TrAl-dv 3p). × 100.

31 Section through the labyrinth shown in figures 25 and 25a, showing the distribution of pigmented and unpigmented tissue. × 100.

27



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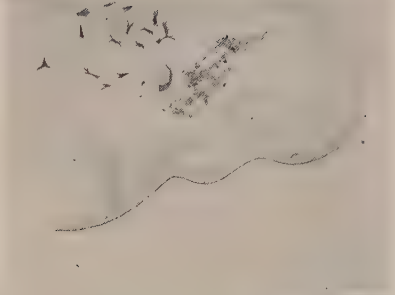

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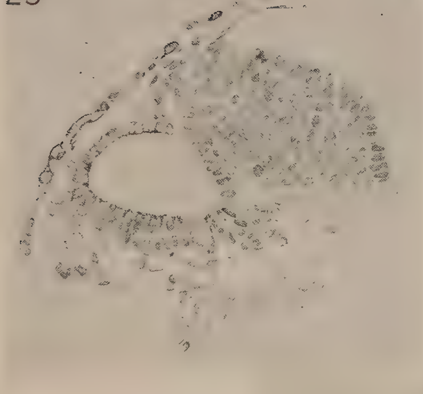

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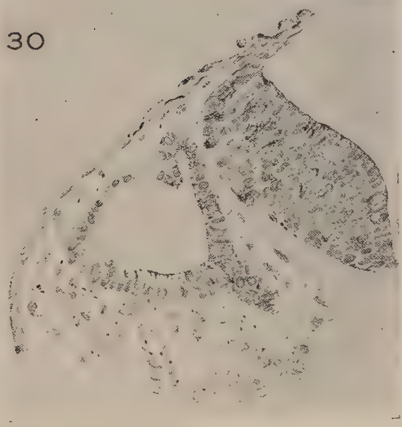

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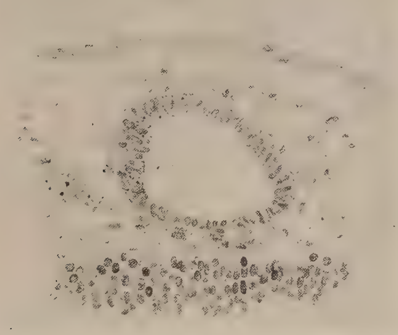

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SYMBIOSIS IN PARAMECIUM BURSARIA

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INTRODUCTION

Although De Bary(1), when he first introduced the term 'symbiosis' into biological literature, broadly defined it as the living together of dissimilar organisms, at the present time the term is used in a variety of ways and to describe a great diversity of conditions. An analysis of the relation between any two symbionts involves not only a consideration of the beneficial relation of one to the other, but also in how far this association may be in any sense a mere commensalism or in any way parasitic. The evaluation of the benefits derived by each of the symbionts from this association constitutes a problem of considerable interest, and one to which, it is believed, the experiments recorded in the present paper make some contribution.

In the case of Paramecium bursaria, the observations will show that the green alga (probably Chlorella vulgaris), when present, contributes in some way to the sustenance of the animal. It will also be seen that the infecting alga in the life-history of a single culture of paramecia may function not only as a true symbiont in the most limited sense, but also as a commensal, and even, under certain conditions, as a parasite.

The author wishes to acknowledge his indebtedness to Prof. Lorande Loss Woodruff, at whose suggestion this work was begun, and to Prof. J. W. Buchanan for his valuable advice throughout the work.

PROBLEM, METHODS, AND RESULTS

It was first noticed that cultures which were seeded from the original culture[1] were in good condition long after it was necessary to renew the food supply of other species of Paramecium, and that they continued to thrive even after the media became quite devoid of organic food material. Evidently, the organisms were deriving nourishment from some source other than the bacterial flora of the water. The fact that algae were present in these organisms naturally suggested the probability that these plant cells were in some way involved in the metabolism of the Paramecium. The problem is to determine the nature of this relationship.

Pringsheim(2), approaching the problem from a different angle, succeeded in rearing animals of this species in a medium free from all organic food material. He prepared a sterile inorganic nutrient solution to which he transferred Paramecium bursaria which had been thoroughly washed free from bacteria. In this medium, from two animals, he raised a rich culture of several hundred individuals. Although no organic food was available for the animal cell, yet everything was supplied for the well-being of the plant bodies within. Pringsheim concluded ''dass Paramecium Bursaria von seinen Zoochlorellen ganz und gar ernährt werden kann und der Aufnahme geformter oder gelöster organischer Stoffe von aussen zu seinem Gedeihen nicht bedarf.'' Thus, it is indicated that the animals depend in some measure at least upon the algae, but the exact rôle of the infecting plant cells was open to further investigation.

In studying this relationship, the observations and data at hand will be weighed in the light of four possible types of association between plants and animals.

A. That the paramecium contributes to the food of the algae

In order to ascertain whether or not the paramecium contributes to the food of the algae, an experiment was devised to determine whether the CO_2 given off by the animal in respiration was utilized by the algae, and, if so, under what conditions. This was done by measuring the amount of CO_2 produced by the paramecia under two diverse conditions, namely, light and darkness.

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[1] The races of Paramecia used in these studies were started from pedigree cultures collected by Prof. L. L. Woodruff.

The determinations were made by the colorimetric method of H-ion determination, using phenolsulphonphthalein, 0.01 per cent, as indicator, and a series of standard colorimetric solutions for comparison.

Thirty small test-tubes of the same caliber, which had previously been chemically cleaned and stored in alcohol, were dried and used for the experiment. In each tube were placed 5 cc. of the culture, which was shaken five times immediately before each sample was taken out. This was done to secure approximately the same number of individuals in each unit. A clean glass bead had been placed in each tube to facilitate stirring at the time when the indicator should be added. As the cultures were added to the tubes they were placed alternately in two groups, one group which was placed in darkness, the other which was left in the light. Hence, the two sets of fifteen tubes each were filled at approximately the same time. Ten samples of 0.1 cc. each were withdrawn from the stock culture at different times while the tubes were being filled, and the number of animals in each sample was counted under the binocular microscope. These samples showed an average population of 19.6 for each 0.1 cc., making an average of about 1000 animals for each 5 cc.

Ten drops of the indicator were added to the first tubes in each group as soon as the sets had been made up. A layer of melted paraffin was poured on the surface of the culture. As a check, a few cubic centimeters of the indicator alone was added next and another layer of paraffin added. After shaking to insure even distribution of the indicator, the pH was read, using the standard tubes for comparison. Thereafter, the indicator was added to the remaining tubes of each set at intervals of thirty minutes each, and the pH read as before. Thus, to tube 15, for example, no indicator was added until seven hours after the series had been started. The pH value for each tube to which the indicator had been added was reread each hour until the completion of the experiment.

The readings are given in tables 1 and 2. By comparing the two series, it will be seen that the H-ion concentration increased more rapidly in the case of the animals kept in darkness, and that, in this series, it was much higher at the end of the experiment. Also, it will be noted that the addition of the indicator had no effect upon the processes under consideration. Culture tubes to which the indicator had been added early in the experiment showed approximately the same increase in H-ion concentration as those which had been without the indicator over the same period of time. For example, tube 13, table 2, showed approximately the same pH at 5.15 as the other five tubes which were read at the same time and to which the indicator had been added at different time intervals. Examination of the data shows this to be substantially true in all cases.

TABLE 1

Carbon-dioxide production of Paramecium bursaria in light

TUBE NO.	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH
1	11.05	7.4	12.05	7.35	1.05	7.3	2.05	7.2	3.05	7.1	4.05	7.05	5.05	7.0
2	11.35	7.2	12.35	7.15	1.35	7.05	2.35	7.0	3.35	7.0	4.35	7.0	5.35	7.0
3	12.05	7.2	1.05	7.1	2.05	7.0	3.05	7.0	4.05	7.0	5.05	7.0	6.05	7.0
4	12.35	7.2	1.35	7.1	2.35	7.1	3.35	7.1	4.35	7.1	5.35	7.0	6.35	7.0
5	1.05	7.1	2.05	7.05	3.05	7.0	4.05	7.0	5.05	7.0	6.05	7.0		
6	1.35	7.1	2.35	7.05	3.35	7.05	4.35	7.0	5.35	7.0	6.35	7.0		
7	2.05	7.2	3.05	7.15	4.05	7.1	5.05	7.1	6.05	7.0				
8	2.35	7.1	3.35	7.05	4.35	7.0	5.35	7.0	6.35	7.0				
9	3.05	7.1	4.05	7.05	5.05	7.0	6.05	7.0						
10	3.35	7.1	4.35	7.0	5.35	7.05	6.35	7.0						
11	4.05	7.1	5.05	7.1	6.05	7.05								
12	4.35	7.1	5.35	7.1	6.35	7.0								
13	5.05	7.15	6.05	7.1										
14	5.35	7.1	6.35	7.0										
15	6.05	7.1												

As a check on the experiment recorded above, a second experiment was planned to detect any possible difference between the production of CO_2 in light and in darkness when the above experiment was carried out, using colorless instead of green animals. For this study a pedigree culture of Paramecium calkinsi was used. The method was the same as that described above. However, only ten cultures were prepared—five being placed in darkness and five remaining in the light. The population of this culture was roughly estimated by counting the animals in twenty samples of 0.1 cc. each. It was found that the culture averaged 100 animals per cc., or an average of 500 animals for each tube.

TABLE 2

Carbon-dioxide production of Paramecium bursaria in darkness

TUBE NO.	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH
1	11.15	7.2	12.15	7.1	1.15	7.0	2.15	7.0	3.15	6.9	4.15	6.85	5.15	6.85
2	11.45	7.1	12.45	7.0	1.45	6.9	2.45	6.9	3.45	6.8	4.45	6.7	5.45	6.6
3	12.15	7.1	1.15	7.0	2.15	6.9	3.15	6.9	4.15	6.85	5.15	6.85	6.15	6.7
4	12.45	7.05	1.45	7.0	2.45	6.9	3.45	6.8	4.45	6.8	5.45	6.8	6.45	6.7
5	1.15	7.05	2.15	7.0	3.15	7.0	4.15	6.85	5.15	6.85	6.15	6.7		
6	1.45	7.1	2.45	7.1	3.45	7.0	4.45	7.0	5.45	6.9	6.45	6.7		
7	2.15	7.0	3.15	6.9	4.15	6.8	5.15	6.8	6.15	6.6				
8	2.45	6.9	3.45	6.85	4.45	6.8	5.45	6.7	6.45	6.7				
9	3.15	6.9	4.15	6.85	5.15	6.8	6.15	6.6						
10	3.45	7.0	4.45	6.9	5.45	6.8	6.45	6.7						
11	4.15	6.85	5.15	6.8	6.15	6.6								
12	4.45	6.85	5.45	6.8	6.45	6.6								
13	5.15	6.8	6.15	6.6										
14	5.45	6.8	6.45	6.7										
15	6.15	6.8												

The readings are recorded in tables 3 and 4. By comparison of the two, it will be seen that the increase in the H-ion concentration over the period recorded was approximately the same in the two sets. Also, it should be noted that after a period of fifteen hours had elapsed from the time of the last reading recorded in the tables the pH value had not decreased below 7.0 in any case. At that time it was found by examining the tubes under the microscope that the animals were still quite normal.

It is seen from these experiments that the amount of CO_2 given off in darkness by the green animals is much in excess of that given off in the light. Due to photosynthetic activity, the greater part of the CO_2 liberated by the animal in the light must have been utilized at once by the plant cell, for there was no evidence of increase in the acidity of the culture medium. In darkness, apparently no appreciable absorption of the CO_2 by the algae occurs. This is shown by the increase in the H-ion concentration of the culture medium in darkness.

TABLE 3

Carbon-dioxide production of Paramecium calkinsi in light

TUBE NO.	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH
1	12.35	7.4	1.35	7.3	2.35	7.2	3.35	7.15	4.35	7.1	5.35	7.1
2	1.05	7.4	2.05	7.3	3.05	7.2	4.05	7.1	5.05	7.1		
3	1.35	7.3	2.35	7.2	3.35	7.1	4.35	7.1	5.35	7.1		
4	2.05	7.3	3.05	7.2	4.05	7.15	5.05	7.1				
5	2.35	7.25	3.35	7.2	4.35	7.1	5.35	7.1				

Certain observations also indicate that in the absence of light the algae derive some sustenance from the host. Fresh hay infusions were prepared, seeded with green animals and placed in darkness. After some weeks, the animals became quite colorless, but continued to live and multiply, their environment being rich in bacteria. Since some weeks passed before the disappearance of the algae was noticeable, it seems probable that the Chlorella depended upon the host in some way over this period.

Although there may be at times in the life-history of a normal culture of Paramecium bursaria conditions which are so unsuited for the existence of the plant bodies within that entire dependence on the host is necessary, this is not to be considered a normal relationship.

B. That the algae contribute to the food of the paramecium

Green algae require CO_2. By eliminating the CO_2 from the air which was supplied to the culture and by washing the CO_2 from the culture itself by means of a continuous stream of CO_2-free air, the photosynthetic activity of the infecting algae was reduced to a minimum. That the algae contribute to the food of the paramecium was indicated by the behavior of the animals under these conditions.

For this experiment samples of the same culture of P. bursaria were used which was used in the previous experiment. It should be stated that this flask of animals, although in good condition, appeared to contain little, if any, organic food material. Inoculation of various bacteriological media showed the culture to be extremely low in bacterial content. The food had not been replenished nor the medium in any way changed since August, 1924, when the culture was first seeded. The culture then, at the time of the experiment, was a year and a half old, with no renewal of food.

TABLE 4

Carbon-dioxide production of Paramecium calkinsi in darkness

TIME NO.	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH	TIME	pH
1	12.45	7.4	1.45	7.25	2.45	7.2	3.45	7.1	4.45	7.0	5.45	7.0
2	1.15	7.4	2.15	7.2	3.15	7.1	4.15	7.1	5.15	7.05		
3	1.45	7.3	2.45	7.2	3.45	7.1	4.45	7.1	5.45	7.1		
4	2.15	7.2	3.15	7.15	4.15	7.1	5.15	7.1				
5	2.45	7.2	3.45	7.15	4.45	7.1	5.45	7.1				

The apparatus for the elimination of CO_2 consisted of a series made up of the following units: soda-lime tube, two Erlenmeyer flasks containing KOH, another containing distilled water, and the culture, 25 cc., contained in a large test-tube. A water suction pump served to draw the CO_2-free air through the culture.

It was found that, in the case of the culture of P. bursaria exposed to the CO_2-free air, all life was extinct at the end of five days. A pedigree culture of colorless animals (P. aurelia) was placed in the apparatus and subjected to the CO_2-free air. The organisms were in good condition at the end of five days. Comparable results were obtained when a large quantity of the culture (150 cc.) was subjected to a similar stream of CO_2-free air, except that it took twice as long to kill the organisms. The culture was placed in this instance in an Erlenmeyer flask. Another distilled water unit was added as well as a flask of $Ba(OH)_2$ between the KOH and the distilled water. The $Ba(OH)_2$ gave no evidence by the formation of a thin film of $BaCO_3$ of any CO_2 in the air passing over it.

To test whether the green animals succumbed because they may have been more delicate and could not withstand the agitation set up in the apparatus, a third test was made. It was found that the same culture of green animals would survive the period when the air was washed with distilled water only, the KOH units being replaced by water units. In this way the culture was subjected to the same mechanical agitation, but CO_2 was available. Most of the original population survived a ten-day treatment.

Hence, it is evident that the animals infected with algae under these conditions could not supply CO_2 in sufficient quantity for the maintenance of photosynthetic activity on the part of the plant cells. And since the animals in the absence of an external supply of organic food were dependent upon the algae, they starved under the conditions of the experiment.

C. That the paramecium is deleterious to the life of the algae

On February 15, 1925, a flask of fresh sterile hay infusion was inoculated with P. bursaria from the original culture. On April 4th it was noticed that a large number of the animals in two separate flasks made up at the same time had become quite colorless, presenting a marked contrast to the others. By April 22nd the population in the two flasks consisted largely of the colorless variety. Subcultures of fresh hay infusion were inoculated from these colorless animals.

In a very short time they had returned to the green condition and the animal body had become quite filled with algae.

This shows that, under some conditions, the infecting organisms may be digested by the host. As light was supplied, they could not have merely 'bleached,' and all of the population was not so affected. When these colorless animals were transferred to a fresh hay infusion, the few residual alga cells multiplied and the animals resumed their green coloration. However, this is not to be regarded as a common phenomenon in the life-history of a normal culture.

D. That the algae are deleterious to the life of the paramecium

For the sake of completeness, it may be mentioned here that there is no evidence in any of the writer's observations that the infecting algae are in any way injurious to the host.

DISCUSSION

Defining symbionts as organisms living with a host in such a relation that both are benefited(3), then Paramecium bursaria and Chlorella vulgaris are, under certain conditions, true symbionts. For these experiments show that, while the algae contribute to the food of the paramecium, the paramecium also contributes to the food of the algae. This is a relationship of mutual benefit and one which prevails in a culture of animals which is more or less devoid of organic food material. This is in close alignment with the work of Pringsheim(2), who succeeded in getting P. bursaria to live and multiply in a sterile inorganic nutrient medium, and of Keeble(4), who showed that the planarian worm, Convoluta roscoffensis, after it had taken up free algae, was able to live and grow in sea-water which contained only a trace of organic food material. Keeble claimed that the plant cells were able to decompose carbon dioxide and form carbohydrate for the utilization of the animal. The worm could not maintain itself without the green algae and it failed to grow in darkness. Inasmuch as this worm possesses no excretory system, he concluded that the waste products of its nitrogenous metabolism were utilized by the algae.

Defining commensals as organisms which live with a host without benefit or injury to the latter, but to their own advantage(3), then the infecting algae can also be accurately described as commensals, for the results of the CO_2-output experiments show that the algae utilize the CO_2 produced by Paramecium bursaria in the presence of light. Animals when placed in the dark where the photosynthetic factor is eliminated and where plenty of organic food has been supplied continue to live. This indicates that the presence of chlorophyll is not essential for the maintenance of life in P. bursaria, for in the presence of an adequate external food supply paramecia thrive in the absence of photosynthesis.

That the symbiotic algae are also capable of independent existence is shown by the work of Beijerinck(5) who, in 1890, isolated the green bodies from Hydra viridis and succeeded in securing pure cultures of them in artificial medium. He identified these green bodies as Chlorella vulgaris, the form which infects P. bursaria. It is evident from the writer's results and from the work of Beijerinck that the association of P. bursaria and C. vulgaris is not obligatory for the maintenance of life in either.

But the infecting algae are in a precarious position; they must ever be liable to digestion by the host. It seems quite probable that this took place in the case of the culture in which many of the individuals spontaneously lost their algae. This may result from a lack of sufficient inorganic constituents in the old culture medium to satisfy the requirements of the algae. Boschma(6) has described such a phenomenon in the association between Anthozoa and Zooxanthellae. His observations and experiments on a large number of forms indicate that "the association of zooxanthellae with the forms studied is substantially a case of parasitism in which the polyps feed on the algae. The algae probably derived some profit from living in the tissues of the polyps (they are more or less sheltered, and in a position favorable for the action of light, which they need for assimilation; perhaps they may also use the nitrogenous waste products of the polyps), but the final result of the association is that a large number of the zooxanthellae are digested by the coelenterates."

In view of these observations, it does not seem that we are justified in ascribing all phases of the association between Paramecium bursaria and Chlorella vulgaris to symbiosis in the limited sense. Under certain conditions, there exists in a culture of P. bursaria a relationship which can quite accurately be so described. This is characteristic of a culture in which external organic food is either insufficient or lacking. However, the green algae in general live with the host as commensals without either benefit or injury to the latter, but to their own advantage. This association approaches true parasitism when the animals are kept in darkness in the absence of external food and in those cases in which the algae are digested by the host.

SUMMARY

1. In Paramecium bursaria, the infecting algae (Chlorella vulgaris) utilize the CO_2 produced by the animal in the presence of light.
2. The presence of chlorophyll is not essential for the maintenance of life in P. bursaria afforded an adequate external food supply.
3. P. bursaria deprived of an external supply of organic food die when the culture is exposed to a continuous stream of CO_2-free air over a period of time.
4. Colorless animals (P. aurelia) survive a similar exposure to CO_2-free air.
5. In the absence of light, the algae derive some sustenance from the host.
6. The infecting organisms may, under certain conditions, be digested by the host.

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THE SYMMETRY RELATIONS OF TRANSPLANTED LIMBS IN AMBLYSTOMA TIGRINUM

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TWENTY-TWO TEXT FIGURES

INTRODUCTION

As a result of his experiments on limb transplantations in Amblystoma punctatum Harrison shows ('18, '21), that, although the limb bud as a whole is a self-differentiating system, the symmetry relations of the limb are dependent upon its orientation with reference to the axes of the embryo in such a way that, while the polarity of the anteroposterior axis of the limb bud is irreversibly determined at the period of development at which the transplantations are made, the dorsoventral axis is not yet determined, at least not irreversibly. The facts on which these conclusions are based have been stated in the following rules ('21, p. 4):

1. A bud that is not inverted (dorsodorsal) gives rise to a limb of the side of origin of the bud, whether implanted on the same or the opposite side of the body.
2. An inverted bud (dorsoventral) gives rise to a limb of reversed asymmetry, whether implanted on the same or on the opposite side of the body.
3. When double limbs arise, the original one (the one first to begin its development) has its asymmetry fixed in accordance with rule 1 or 2, while the other is the mirror image of the first.

On the other hand, in 1924 Brandt published the results of exactly similar experiments on Triton taeniatus embryos, showing that after transplantation in the tail-bud stage, the asymmetry of the limb was never reversed, while after transplantations in neural-fold stages, the limbs developed according to Harrison's rules. The dorsoventral axis in Triton taeniatus consequently becomes irreversibly determined at a much earlier stage of development than it does in Amblystoma punctatum.

During the years 1922 to 1925 Gräper published a series of papers: "Extremitätentransplantationen an Anuren I–V," in which also the same problems of symmetry relations are discussed. Gräper transplanted the hind legs in several species of anuran larvae, but in comparatively advanced stages of limb development, the length of the limb bud varying from half the diameter of the bud up to twice the diameter. Gräper did not, except in his later experiments, pay attention to the orientation of the graft, which Harrison's experiments have shown to be of vital importance in the determination of the asymmetry of the limb. For these and still other reasons, such as the relatively small size of the grafts and the difficulty of cleaning the wound beds, Gräper's experiments cannot very well be compared with those of Harrison, and cannot be expected to give similar results. As to the symmetry relations Gräper also found in orthotopic transplantations: "dass es möglich ist, die Seitenbestimmung der Hinterbeinsknospen bis zu einer Grösse von 1.5/1 durch Transplantation auf die entgegengesetzte Seite desselben oder eines anderen Tieres ins Gegenteil zu verkehren, so dass nunmehr eine zum Wirt seitenrichtige Extremität entsteht" ('22, I, p. 301). This most probably was after heteropleural dorsoventral transplantations, where also, according to Harrison's rules, a normal[1] limb should be expected. But Gräper apparently means that reversal of the asymmetry in his anuran larvae occurs only where it may result in the outgrowth of a normal limb.[2] He considers it consequently merely as a form of regulation, in which respect his view differs from that of Harrison.

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[1] Gräper's "wirtsseitenrichtig."

[2] In his last paper (V, '25, p. 5) Gräper mentions that he has not succeeded in getting reversed limbs after homopleural dorsoventral transplantations, all the positive cases resulting in reduplications.

Since it is quite possible that the time of the final determination of the polarity of the axes may vary even among comparatively closely related species, Professor Harrison suggested to me to do the same kind of limb transplantations on Amblystoma tigrinum as he had formerly done on A. punctatum. Another reason for choosing this species was that the limbs in A. tigrinum appear at a later stage of development than in A. punctatum. A limb bud transplanted in the tail-bud stage of A. punctatum begins to grow about stage 36, which is shortly after the operation, while in A. tigrinum it will not begin to grow until about ten days later. During this time it might be subject to possible regulatory influences from the host in a much higher degree than the actively growing limb of A. punctatum.

I feel very much indebted to Professor Harrison for advice and suggestions during the work.

The experiments were carried out during a stay at the Osborn Zoölogical Laboratory, Yale University, made possible by the award of the International Fellowship of the American Association of University Women.

The sketches used in this article were all redrawn by Miss Ida Rader, the artist of the zoölogical laboratory in Oslo.

MATERIAL. METHODS. GENERAL FEATURES OF GROWTH

All experiments were made upon embryos of Amblystoma tigrinum at stages corresponding to stages 32–33 in A. punctatum, after the method described by Harrison ('21, p. 5, *a.f.*), whose terminology will be used. The same eight categories of operations were performed. Of these all the orthotopic transplantations were made upon embryos from eggs that had been sent from Chicago, Illinois; that is, eggs from metamorphosed animals, while all the heterotopic transplantations were made upon embryos of the axolotl form kept in aquaria in the laboratory. The total number of cases of which records have been kept is 135, of which only thirty-seven yielded positive results, owing chiefly to the large number in which resorption of the graft took place.

As already mentioned, the fore limbs of Amblystoma tigrinum hardly begin to show until the yolk is resorbed; that is, until a stage of development is reached at which, in A. punctatum, the fore limbs are fully developed. On A. tigrinum at this stage they are still only small nodules just below the pronephros. As they begin to grow they point dorsoposteriorly with the radial border of the hand ventrolaterally, the third and fourth digits appearing on the dorsomedial border. The palmar surface of the hand faces ventromedially. In these respects the development in the two species is similar.

The transplanted limbs follow the same course of development as Harrison found in Amblystoma punctatum; that is, if the anteroposterior axis of the bud is not inverted by the transplantation—homopleural dorsodorsal and heteropleural dorsoventral transplantations—the growing limb will point posteriorly or dorsoposteriorly like a normal limb. If the anteroposterior axis of the bud is inverted by the transplantation, as in the homopleural dorsoventral and heteropleural dorsodorsal transplantations, the outgrowing limb will point anteriorly. In the orthotopic transplantations there was a distinct difference between the homopleural dorsoventral and heteropleural dorsodorsal transplantations with respect to the outgrowth of the limb. After the latter operation the limbs usually grew straight forward under the operculum ventral to the gills, while the homopleural dorsoventral transplants showed a tendency to grow more dorsally close behind and under the roots of the gill filaments. If they were not very soon released from this position—that is, forced down below the gills or out between them—they usually formed only abortive limbs. The heteropleural dorsodorsal grafts usually developed into fully differentiated limbs, even if they remained for quite a long time under the operculum, the resulting single limb lying close to the head, with the palmar surface of the hand toward the head. The homopleural dorsoventral (single reversed) limbs very often stuck out more or less sharply to the side with the palm facing more anteriorly and dorsally.

HETEROTOPIC TRANSPLANTATIONS

In all of these experiments the limb bud was implanted on the right flank of the embryo.

Table 1 gives a summary of the results of the transplantations. Sixty-five experiments were made, out of which only eleven (17 per cent) yielded positive results.

TABLE 1

Heterotopic transplantations. Summary of experiments

	NUMBER OF EXPERIMENTS	DIED PREMATURELY	GRAFT RESORBED	ABNORMAL LIMBS	POSITIVE	SINGLE LIMBS REVERSED	SINGLE LIMBS NOT REVERSED	REDUPLICATIONS
Hom. dd.	11	9			2		2	
Hom. dv.	20	12	6	1	1		1[3]	
Het. dd.	18	13	1[1]		4		4	
Het. dv.	16	7	2	3	4	2[2]	2	
	65	41	9 (37.5%)	4	11	2 (18%)	9 (82%)	

[1] Resorbed after having grown out to the bidigitate stage, perhaps broken.

[2] One of these cases (A.t. 171) was intended to have been oriented dorsodorsally. However, it showed pronephric tubules on the ventral side of the growing limb, which therefore must have been inverted by mistake. As the notes on the donor record that its left fore limb was lacking, the case must have been a het. dv., and it is therefore tabulated among those. It also grew out posteriorly from the beginning.

[3] Forward pointing and upside down.

Of Harrison's 138 heterotopic transplantations, forty-five (33 per cent) were positive. This difference is certainly due partly to the more pronounced tendency for the graft to be resorbed in A. tigrinum. However, most of the experiments were done in New Haven in May–June, 1925, and the first week of June was extraordinarily hot. The larvae apparently suffered from the heat and would not take any food for more than a week. On this account the grafts most probably developed less than they would have under more favorable conditions.

1. Homopleural dorsodorsal orientation (*hom. dd.*)

Out of these transplantations only two survived, and in both of them the transplanted limb from beginning to end developed like the normal limbs into a limb of the same side (fig. 1).

2. Homopleural dorsoventral orientation (*hom. dv.*)

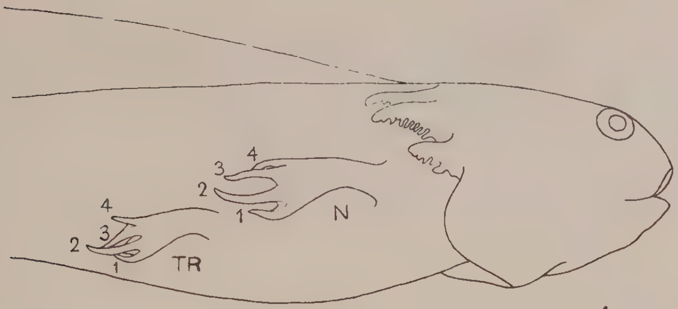
Twenty cases were operated upon in this way. One developed an abortive limb, a stump about half the length of a normal arm, pointing straight out to the side. In only one case the graft developed into a normally differentiated limb (A.t. 192). The initial direction of pointing was here ventroanterior, that is exactly the opposite of the normal. The third and fourth fingers were developed on the ventromedial side of the hand, which has the palm turned in toward the body; that is, the limb is a right limb upside down and forward pointing. It has consequently developed in the inverted position (fig. 2). Here six out of eight cases resorbed the graft.

3. Heteropleural dorsodorsal orientation (*het. dd.*)

Eighteen operations were done in this series. Thirteen died prematurely, four developed single limbs preserving their prospective asymmetry (fig. 3). In another one the transplanted limb developed initially just like these, pointed anteriorly, and grew out to the bidigitate state, then it became sharply bent downward—perhaps broken—and disappeared entirely during the following week.

4. Heteropleural dorsoventral orientation (*het. dv.*)

Sixteen experiments of this kind have been made. Seven died too early, and in two the graft was resorbed; three de-



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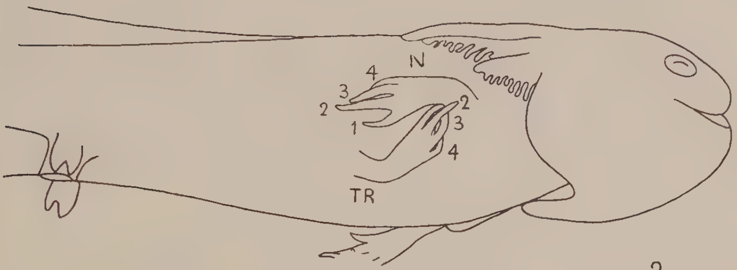

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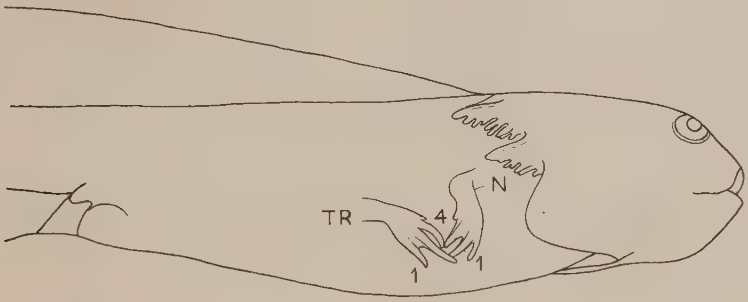

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Fig. 1 Experiment A.t. 187. Heterotopic transplantation; right fore limb to right flank (hom. dd.). Lateral view, drawn from preserved specimen, killed fifty-nine days after operation. *TR,* transplanted limb; *N,* normal right fore limb; *1* to *4,* first to fourth digits. × 8.

Fig. 2 Experiment A.t. 192. Heterotopic transplantation, right fore limb to right flank, inverted (hom. dv.). Lateral aspect, drawn from specimen preserved fifty-eight days after operation. Transplanted limb (*TR*) has developed in inverted position. × 8.

Fig. 3 Experiment A.t. 240. Heterotopic transplantation, left fore limb to right flank (het. dd.). Lateral view, drawn from specimen preserved fifty-five days after operation. Transplanted limb (*TR*) has retained its original asymmetry. × 8.

veloped abnormal limbs, the asymmetry of which could not be determined. In two cases[3] the transplanted bud grew out very much like the normal limb bud of the same side, pointed only a little more laterally than the normal one did, and developed into an unquestionable right limb (fig. 4). In these cases, consequently, the limbs had had their asymmetry reversed.

In two other cases the transplanted limbs retained their original asymmetry, remaining left limbs upside down. In both of them the hand is almost horizontal. In figure 5 the limb sticks out sharply to the side, the posterior border is the ulnar border and the palmar surface of the hand is turned upward. In the other case the limb is pressed more closely in toward the body wall, pointing posteroventrally, with the third and fourth digits on the ventromedial side of the hand and the palm turned in toward the body.

SUMMARY OF THE RESULTS OF THE HETEROTOPIC TRANSPLANTATIONS

Surveying the dorsodorsal transplantations in table 1, we see that the implanted limb buds always give rise to limbs that retain their original asymmetry, exactly as was the case in Harrison's transplantations on Amblystoma punctatum.

Of the four positive cases among the heteropleural dorsoventral orientations, two retained their original asymmetry and two became reversed, while according to the rules we should have expected them all to become reversed.

Gräper states that a certain retardation in the outgrowth of the transplanted limbs is necessary, if the asymmetry is to be reversed. Now the outgrowth of the limbs in Amblystoma tigrinum is normally very much retarded compared with Amblystoma punctatum. If, therefore, the rate of growth were the only factor of importance for the reversal of asymmetry, we should naturally expect a higher percentage of reversed limbs in tigrinum than in punctatum. That does not seem to be the case; in fact, in Harrison's experiments on

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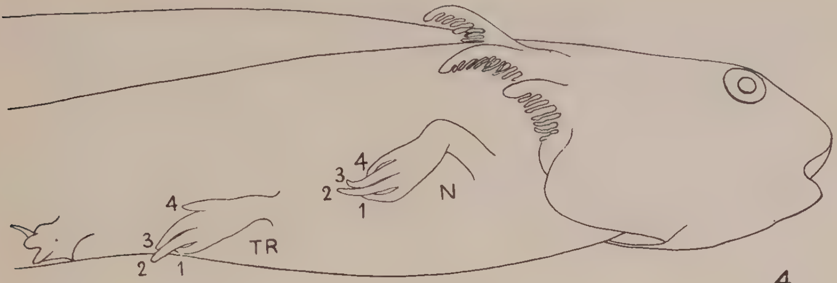
[3] Note 2 to table 1.



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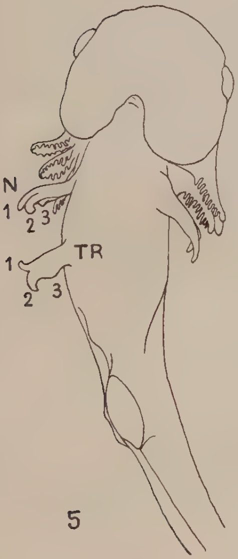

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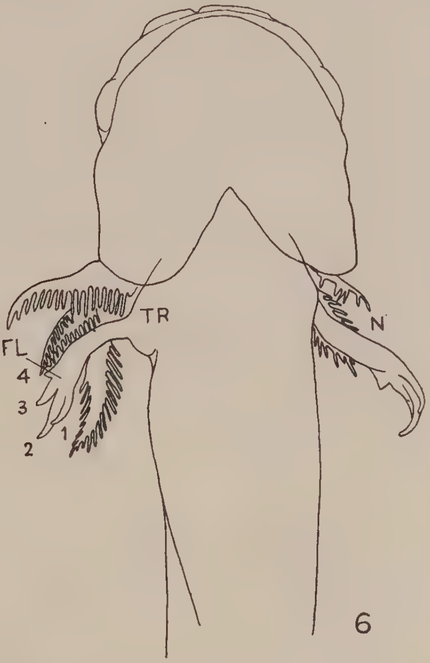

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Fig. 4 Experiment A.t. 231. Heterotopic transplantation; left fore limb to right, flank inverted (het. dv.). Lateral view drawn from specimen preserved fifty-five days after operation. Transplanted limb (*TR*) has developed into a right limb; i.e., has had its asymmetry reversed. × 8.

Fig. 5 Experiment A.t. 189. Heterotopic transplantation, left fore limb to right flank inverted (het. dv.). Ventral aspect, drawn from specimen, preserved in dying condition forty-six days after operation. As the palmar side of the hand is facing dorsally, the transplanted limb (*TR*) is a left limb. × 8.

Fig. 6 Experiment A.t. 52. Orthotopic transplantation; right fore limb to right side inverted (hom. dv.), resulting in a left limb on right side. Ventral aspect drawn from specimen, preserved fifty-nine days after operation. *N*, normal left limb; *TR*, transplanted limb; *FL*, palmar side of the hand facing anteroventrally. × 8.

punctatum there was only one supposed case out of sixteen heteropleural dorsoventral transplantations that did not have its asymmetry reversed, and this one was most probably due to an experimental error.

With regard to the rate of growth in the four tigrinum heteropleural dorsoventral transplantations, the grafted limb in no case was considerably retarded compared with the normal ones. However, the growth of the whole animal in one of the cases with reversed asymmetry was very much retarded (in exp. A.t. 171). This was one of the larvae that refused to take food for more than a week, and it grew all the time very slowly.[4] The three remaining cases, however, grew at very much the same rate all the time, and in spite of that one had its asymmetry reversed and two not.

Although the number of positive cases is small, the probable explanation of the fact that the results are not uniform is rather that stage 32–33 in Amblystoma tigrinum is the transitional stage, when the polarity of the dorsoventral axis of the limb is becoming irreversibly determined, and that this stage is not sharply defined, but is reached by different individuals at slightly different times. Besides, it is impossible always to be perfectly sure about the exact stage of an embryo; the selected embryos may certainly sometimes be a little bit older and sometimes a little bit younger than supposed.

This would imply that the dorsoventral axis of the limb bud[5] becomes irreversibly determined at an earlier stage in Amblystoma tigrinum than in Amblystoma punctatum, where it, in corresponding stages, may still be reversed.

This, however, cannot be definitely determined without further experiments, which I hope to be able to perform in the coming season.

Also the only positive case among the homopleural dorsoventral transplantations remained unreversed, contrary to the rules. Most probably this, then, had been operated upon at a comparatively advanced stage. The limb developed in the inverted position and did not rotate back to the normal posture, as do the limbs in the corresponding orthotopic transplantations that do not become reversed (Harrison, '21).

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[4] It was fixed seventy days after the operation and measured then 20.5 mm., while A.t. 231 (the other case where the asymmetry was reversed) was fixed fifty-five days after the operation, measuring 23 mm.

[5] Gräper's Querschnittdetermination.

Table 1 also shows that the percentage of resorbed grafts is higher among the dorsoventral orientations than among the dorsodorsal. This is in reality even more striking than the table shows, because in the one case of resorption among the heteropleural dorsodorsal transplantations, the limb was not resorbed till it had grown large, the resorption here most probably being due to a different cause, so that this case is probably not parallel to the other resorption cases, but should be classified as an abnormality. As will appear from table 2, the orthotopic transplantations also show a much higher degree of resorption among the dorsoventral transplantations than among the dorsodorsal.

Another striking fact is that not a single reduplication occurred. Even among this small number of positive cases one should have expected a few such, if the tendency to reduplication is as strong in A. tigrinum as in A. punctatum ($33\frac{1}{3}$ per cent).

ORTHOTOPIC TRANSPLANTATIONS

In all of these experiments the right limb bud of the host was removed, the remaining mesodermal cells carefully cleaned off, and a limb bud from another embryo grafted into the wound bed.

Table 2 gives a summary of the results of these transplantations. Seventy operations were made, out of which only twenty-six (37 per cent) yielded positive results. In Harrison's experiments on Amblystoma punctatum, ninety-four out of 145 were positive (65 per cent). The poor result in A. tigrinum is again due to the high proportion of resorbed grafts (43 per cent).

1. Homopleural dorsodorsal orientation (*hom. dd.*)

In all the positive cases the transplanted limb developed into a perfectly normal right limb. As a rule, the transplanted limbs were somewhat smaller than the normal left limbs. In one case, however (A.t. 59) the transplanted limb was a little bit ahead of the left one all the time, and here the record states that the host looked considerably younger than the donor when the bud was transplanted.

TABLE 2

Orthotopic transplantations. Summary of experiment

	NUMBER OF EXPERIMENTS	DIED PREMATURELY	GRAFT RESORBED	ABNORMAL LIMBS	POSITIVE	SINGLE LIMBS REVERSED	SINGLE LIMBS NOT REVERSED	REDUPLICATED
Hom. dd.	15	5	1 (10%)		9		9	
Hom. dv.	30	6	14 (58%)	2	8	3	3	2
Het. dd.	17	6	2 (18%)	1	8		8	
Het. dv.	8	2	5 (83%)		1		1	
	70	19	22 (43%)	3	26	3 (11%)	21 (81%)	2 (8%)

2. Homopleural dorsoventral orientation (*hom. dv.*)

Out of the twenty-four specimens that grew large enough to show the character of the limbs, fourteen resorbed the graft. In one of the two cases which yielded abortive limbs, the bud grew dorsally, close behind the roots of the gill filaments, into a long, thin appendage. Its distal part was curved slightly backward, and it showed a tiny little notch at the tip, as if indicating a differentiation into two very rudimentary digits; no elbow-joint was formed. The other abnormal limb was a short stump without any differentiation, and was distinctly constricted at its base.

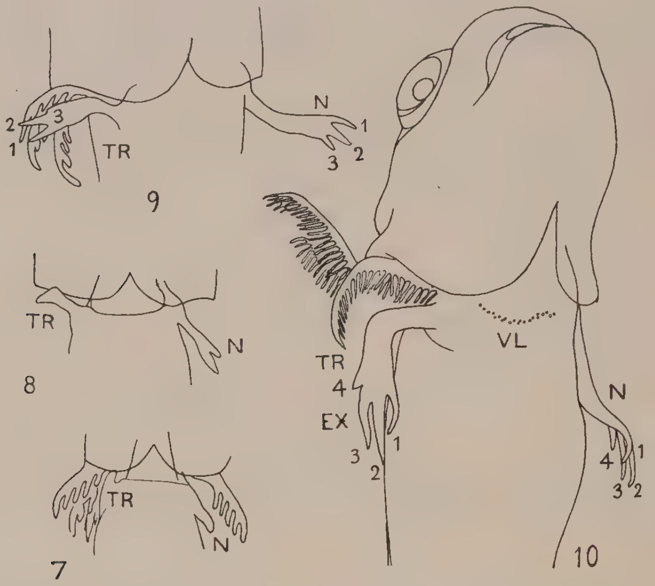
Of the eight positive cases, the transplanted bud in two individuals gave rise to double limbs, and in six to single limbs. Three of the six had had their asymmetry reversed;

i.e., they were left limbs on the right side; the other three were homopleural limbs.

In all those cases where left limbs developed, the limb buds pointed anterodorsally to begin with, then more and more dorsally up behind the gills. After a while they were forced out between the gill filaments, and when fully differentiated they stood straight out from the body. In one case (A.t. 35) the plane of the hand was almost vertical, the ulnar border was dorsal, the elbow bend was turned upward, the palm of the hand forward. In another case (A.t. 52) the plane was almost horizontal, the anterodorsal border was the ulnar border, the elbow-joint turned forward, the palm facing anteroventrally (fig. 6). In the third case (A.t. 73) there was no distinct elbow bend, the posterodorsal border was the ulnar border, and the palm faced anterodorsally. The two last-mentioned positions may be considered as the result of a rotation of the arm, as seen in the first case (A.t. 35), respectively, forward and backward at the shoulder-joint. In A.t. 73 there grew out also a normal right limb ventral to the grafted one. This limb bud did not begin to show until the primary limb was distinctly bidigitate and showed signs of the third and fourth fingers. It grew out at a considerable distance ventral to the shoulder-joint of the primary limb and was, most probably, a regeneration from the host, indicating that the wound bed had not been perfectly cleaned.

Two of the heteropleural limbs (A.t. 35 and 52) were perfectly normal in their function as far as a limb from the opposite side of the body can be said to be so. A.t. 73, however, seemed abnormally stiff and made only very slight movements at the shoulder-joint.

In the three cases where the transplanted limbs developed into homopleural, single limbs, the asymmetry apparently had not been reversed. In A.t. 18 (figs. 7 to 10) the limb bud at first pointed almost straight forward (fig. 7), but then gradually more and more laterally, rotating backward in an almost horizontal plane (figs. 8 and 9). When all the digits were differentiated and the specimen was preserved, the upper arm was pointing straight out from the body, the anterolateral border of the limb was the ulnar border, the palm of the hand was facing upward (fig. 10). Thus the position of the arm, when preserved, was not quite normal. It was twisted outward and downward at the shoulder-joint.



Figs. 7 to 10 Experiment A.t. 18. Orthotopic transplantation; right fore limb to right side inverted (hom. dv.). Transplanted limb (*TR*) has reached an almost normal posture through rotation. *Ex*, extensor (back) side of the hand; *VL*, ventral sensory line of the operated side. Figs. 7 to 9, free-hand sketches from the living specimen.

Fig. 7 Ventral view of the limb region twenty-three days after operation.

Fig. 8 Same, twenty-eight days after operation.

Fig. 9 Same, forty-three days after operation.

Fig. 10 Camera-lucida drawing from specimen, preserved fifty-nine days after operation, ventrolateral view. × 8.

In this case it is quite clear that no reversal of the asymmetry has occurred. The limb has remained a right limb, which, under the influence of its new surroundings, has rotated backward toward its normal position, but has not reached it.

The two other cases, however, where reversal of the asymmetry apparently did not occur, most probably are due to reduplications, where the original member of the pair was completely reduced. This shows very distinctly in figures 11 to 13 (A.t. 30), but a little less so in figures 14 to 20 (A.t. 25).

In A.t. 30 the transplanted limb bud at first pointed anterolaterally, and was very large with a broad, square tip. Twenty days after the operation, when the left limb was slightly bidigitate, a secondary bud had begun to grow out from the ventroposterior base of the transplanted limb bud (fig. 11). This secondary bud grew backward and developed into a slightly atypical right limb, while the forward-pointing part of the double bud was completely reduced (figs. 12 and 13). It is here perfectly clear that it was a secondary bud that developed, and as this is an ordinary right limb, the primary bud, according to the mirroring rules, must have been a left one; that is, it had had its asymmetry reversed.

The reduplication process does not show so clearly in figures 14 to 20 (A.t. 25). Here also the original direction of pointing of the limb bud was anterior (figs. 14 and 15), while later on the limb grew backward (figs. 16 to 20). In this case the origin of the posterior secondary bud was not actually observed and the change between the stages on figures 15 and 16 is rather abrupt. Comparing it with the former case, however, I am inclined to consider it as belonging in the same category.

Among the six cases where single limbs developed, there is most probably, then, only one single case in which asymmetry was not reversed. In three out of the five cases in which reversal occurred the transplanted limb developed into a single heteropleural limb, while in two cases, owing to a reduplication process and reduction of the primary (reversed) member of the pair, the secondary member developed into a homopleural limb.

One of the two double limbs (A.t. 28) which resulted in this series was perfectly symmetrical[6] and mirrored in a radial plane through the second digit, whose proximal part was single, while the distal four joints were double. A normal right limb developed ventral to the double one, indicating here also insufficient cleaning of the wound bed. The second double limb consisted of a double hand only with seven digits in all, the identity of which it was impossible to establish.

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[6] What Gräper would have called 'indifferente Fächerbildung.'

3. *Heteropleural dorsodorsal orientation* (*het. dd.*)

Out of the eleven cases which did not die prematurely, two resorbed the graft and one developed an abortive limb, a short somewhat flattened stump without any indication of elbow bend or digits. In the eight really positive cases the transplanted limb always retained its prospective asymmetry and developed into a single left limb on the right side (fig. 21).

The young limb buds always pointed anteriorly, and in most of the cases the limb grew straight forward under the operculum and ventral to the gills. After having been forced outside the operculum, the limbs remained lying with their palms close in to the head. After they had begun to move, they might, of course, be directed in any way.

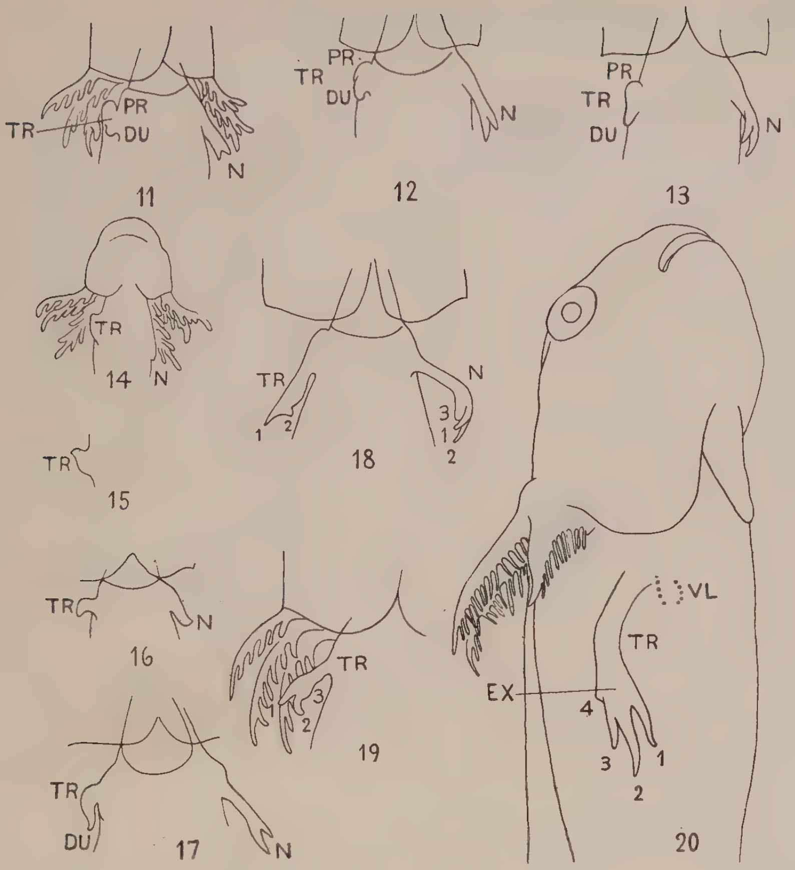
4. *Heteropleural dorsoventral orientation* (*het. dv.*)

In these experiments five specimens out of six resorbed the graft and only a single one gave rise to a well-formed limb. Here the transplanted limb developed in the inverted position into a single, left limb upside down on the right side of the body (fig. 22, A.t. 60).

GENERAL DISCUSSION

We see in the orthotopic transplantations exactly the same condition as shown in the heterotopic transplantations, the transplanted limb bud in all dorsodorsal orientations retained its prospective asymmetry in agreement with Harrison's rules.

As mentioned in the introduction, Gräper seems to hold that in orthotopic transplantations regulatory influences from



Figs. 11 to 13 Experiment A.t. 30. Orthotopic transplantation; right fore limb to right side, inverted (hom. dv.). Reduplication and reduction of the primary bud (*PR*), while the reduplicating member (*DU*) developed into a homopleural limb. Free-hand sketches from the living specimen.

Fig. 11 Ventral view of the limb region twenty days after operation.

Fig. 12 Same, twenty-four days after operation.

Fig. 13 Same, twenty-eight days after operation.

Figs. 14 to 20 Experiment A.t. 25. Orthotopic transplantation, right fore limb to right side, inverted (hom. dv.). Reduplication and reduction of the primary bud(?), resulting in a homopleural limb. *DU*, reduplicating member. *Ex*, extensor (back) side of the hand. *VL*, ventral sensory line of the operated side. Figs. 14 to 19, free-hand sketches from the living specimen.

Fig. 14 Ventral view, eight days after operation.

Fig. 15 Ventral view of the limb, seventeen days after operation.

Fig. 16 Ventral view of the limb region, twenty-one days after operation.

Fig. 17 Same, twenty-six days after operation.

Fig. 18 Same, thirty-six days after operation.

Fig. 19 Same, forty-one days after operation.

Fig. 20 Ventrolateral view of the specimen preserved fifty-seven days after operation. × 8.

the surroundings determine whether reversal of the asymmetry of the limb shall occur or not. He also reports in one case reversal of the asymmetry after heteropleural dorsodorsal transplantation, in which case a normal limb developed.[7] In A. tigrinum, where the rules of symmetry are seldom obscured by reduplication processes, a heteropleural dorsodorsal orientation of the graft never resulted in a homopleural limb (twelve positive cases), not even in the orthotopic transplantations, where regulatory influences, because of the retarded outgrowth of the limbs in A. tigrinum, might operate under especially favorable conditions. The same possible regulatory influences were, on the other hand, in the homopleural dorsoventral orientations unable to prevent the reversal of asymmetry, so that here in half the cases of orthotopic transplantations, where single limbs developed, left limbs arose from inverted right limb buds.

It is thus quite clear, contrary to Gräper's view, that reversal or non-reversal of the asymmetry of limbs in Amblystoma has nothing to do with regulatory processes. The facts at present can best be accounted for on the hypothesis that the transplantations were made in the transitional period in which the polarity of the dorsoventral axis of the bud is being irreversibly determined. If this axis is determined, the limb will develop in the inverted position, even if the result is a limb that is in disharmony with its surroundings. This occurred only once in the orthotopic transplantations, the single positive case among the heteropleural dorsoventral transplantations. Among the heterotopic transplantations we had two such cases among the heteropleural dorsoventral and one in the homopleural dorsoventral orientations. On the other hand, if the dorsoventral axis at the time of transplantation is not irreversibly determined, reversal will take place even if the result is a disharmonic limb, as occurred in the three cases among the orthotopic transplantations with homopleural dorsoventral orientation.

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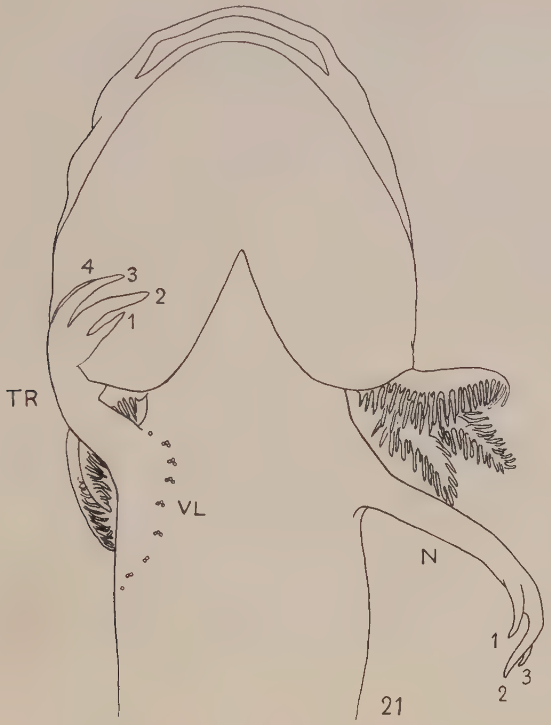
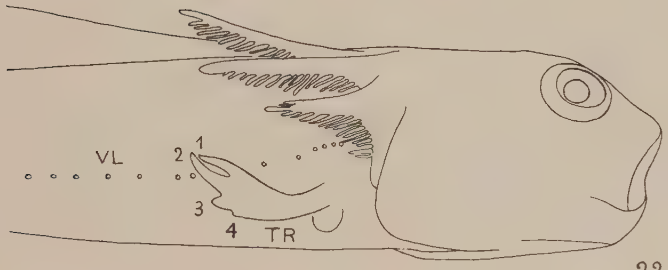
[7] I, '22, p. 304.





22

Fig. 21 Experiment A.t. 61. Orthotopic transplantation, left fore limb to right side (het. dd.). Ventral view of specimen preserved seventy-two days after operation. Transplanted limb (*TR*) has retained its original asymmetry. *VL*, ventral sensory line of the operated side. × 8.

Fig. 22 Experiment A.t. 60. Orthotopic transplantation, left fore limb to right side, inverted (het. dv.). Lateral view of specimen preserved fifty-seven days after operation. Transplanted limb (*TR*) has retained its original asymmetry and developed in inverted position. × 8.

The transitional period, during which this determination takes place in A. tigrinum seems to be about stages 32–33, or somewhat earlier than in A. punctatum, where, according to unpublished experiments of Dr. F. H. Swett, the polarity of this axis is not determined at stage 32, but is definitely determined by stage 35. Thus, in spite of the fact that in Amblystoma tigrinum the fore limb develops at a much later period than in A. punctatum, the asymmetry of the fore limb is determined at an earlier stage than in the latter species.

These facts, therefore, do not support Brandt's ('24) suggestion that the earlier determination of the polarity of the anteroposterior axis of the limb in Triton is correlated with the more precocious external development of the limb itself,[8] for the two species having this precocious development of the fore limb are at opposite extremes with reference to the determination of the polarity of this axis. In Triton taeniatus this determination takes place in the neurula stage, while in Amblystoma punctatum it does not occur until the tail bud is well formed and movement in the embryo has begun. A. tigrinum with its much-delayed external development of the limb is intermediate in respect to its axial determination.

NOTE ON THE VENTRAL SENSORY LINE AFTER ORTHOTOPIC TRANSPLANTATIONS

As will be seen from the figures 6, 10, 20, 21, 22, the ventral sensory line of the operated side in all cases is more or less abnormal. In figure 6, *f.i.*, it is lacking and in figure 22 the line runs backward above the fore limb instead of below. As this last case represents a heteropleural dorsoventral transplantation, where the limb developed in the inverted position, with its ventral border turned upward, one might here say that the ventral sensory line had developed in harmony with the transplanted limb and in disharmony with the host. This fact made me examine more closely the development of the line after orthotopic limb transplantations. And it very soon became evident that the ventral sensory line of the operated side hardly ever was absolutely normal.

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[8] "Der langsamere Ablauf von Entwicklungsvorgängen bei *Amblystoma,* der biologisch in der verspäteten Determination der dorsoventralen Polarität der Gliedmassenknospe in Erscheinung tritt, ist auch äusserlich an der verlangsamten normalen Entwicklung der Extremität erkennbar. Auch diese läuft bei *Triton* schneller ab" (Op. cit. p. 553).

At the stage of operation the placode of the ventral sensory line is situated on a level with the dorsal part of the first somite.[9] The cutting out of the circular limb disc beneath the third, fourth, and sixth somites therefore cannot possibly affect it directly.

It has been shown by Harrison ('03) that irregularities in the integument, such as scars, may cause the sensory lines to change their outgrowth direction (Harrison). And as the placode of the ventral sensory line most probably grows out after orthotopic transplantations through the transplanted material, it would not be surprising if its development were influenced by such transplantations. The question of interest in this connection is now, whether the disturbances in the development of the line is due solely to the operation as such, or if also the orientation of the grafted material may be of any significance for the outgrowth of the line. The course of the ventral line in figure 22 really seems to indicate this.

The examination of my material clearly showed that the irregularities caused by the operation as such consisted in reduction both in length and in the number of individual sense organs, but almost never in abnormal course. About 40 per cent of all cases showed 'reduced' lines, and these were equally distributed over all four categories of experiments.

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[9] Stone's figure 6.

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Dr JOHN HILL

Reprinted from The American Naturalist, Vol. LX,
September–October, 1926

THE VERSATILE SIR JOHN HILL, M.D.

PROFESSOR LORANDE LOSS WOODRUFF
PROFESSOR OF PROTOZOOLOGY, YALE UNIVERSITY

The eighteenth-century in London was a period of contrasts. The town had developed quite suddenly a new method of living, social intercourse being the dominant note with its outlet in drum and rout, coffee house and tavern, Vauxhall and Ranelagh. The ostentatious display of a Chesterfield brought into relief the unkempt personality of a Johnson. On the intellectual side, with all due respect to the Lord and the Doctor, those who have recently been appropriately dubbed the "Fifth Estate"[1] were diligent investigators in a largely unexplored field. The previous century, as exemplified by Robert Hooke's "Micrographia,"[2] had demonstrated the profit of studying nature with mechanical aids to the senses and instruments of precision, and the furrows it turned were being slowly seeded and the first products garnered. The contrast between the man of science and the man of the street was probably never so great—and both extremes were embodied in Sir John Hill, M.D. Dr. Hill is a type which we always have with us, but the London of his day afforded free play for his versatility as apothecary, astronomer, actor, botanist, microscopist, mineralogist, zoologist, physician, editor, journalist and man of fashion. One has but to turn the pages of London's print from 1750 to 1775 to meet his name.

John Hill was the second son of the Rev. Theophilous Hill and was born in 1716 or 1717 either at Peterborough or Spalding. Little is known regarding his early education, except that it did not include formal academic work, and we first find him apprenticed to an apothecary, as was not unusual at the time for those who planned to practice medicine. But lectures on plants which he attended at the Chelsea Physic Garden apparently gave him an unusually broad botanical foundation, and it was this knowledge that he immediately attempted to capitalize when in his early twenties he found the income from his own apothecary shop in St. Martin's Lane unequal to the needs of a growing family. He traveled extensively in England, collecting plants which he dried and arranged in sets with classifications and descriptions. Sufficient subscriptions to the *hortus siccus,* however, were not forthcoming, and after some horticultural work for certain of the nobility he forsook temporarily the profession of herbalist for that of actor.

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[1] A. D. Little, "The Fifth Estate," *Science*, 60, 1924.

[2] L. L. Woodruff, "Hooke's Micrographia," Amer. Naturalist, 53, 1919.

Hill's appearance at the Haymarket and Covent Garden was apparently equally unprofitable from the standpoint of his finances and his reputation, if we may judge from amusing accounts given by his contemporaries; but this did not deter, indeed, probably inspired him a few years later to publish anonymously a stout little volume entitled "The Actor; a Treatise on the Art of Playing, Interspersed with Theatrical Anecdotes, Critical Remarks on Plays, and Occasional Observations on Audiences." And this he dedicated "To the Managers of the Two Theatres." That it attracted attention at the time is clear by the quarrels in which it involved its author, and these were accentuated later by two mediocre plays[3] whose failure Hill attributed to various actors, with whom he exchanged uncomplimentary epigrams. Garrick wrote:

> "For physic and farces his equal there scarce is;
> His farces are physic, his physic a farce is."

Sensing at once that the stage of the time offered nothing, Hill resumed his practice as apothecary and physician, fortified by a medical degree granted, *in absentia*, by St. Andrews. The available evidence indicates that he was not unsuccessful, for in 1746 we find him acting as a regimental surgeon, with time to edit the *British Magazine* and to publish a translation of Theophrastus's "Treatise on Gems" with additions and critical notes which considerably increased its contemporary interest and value. Where Hill secured the knowledge which enabled him to annotate successfully this work, which was revived eight years later in a French translation, remains an enigma, but it is only the first of a long series of surprises that Hill affords as his career proceeds. At all events this essay in the field of science brought him to the favorable attention of certain eminent fellows of the Royal Society, in particular Martin Folkes, president of the society, and Henry Baker,[4] the well-known microscopist, who for a time accorded him their patronage.

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[3] "The Maiden's Whim" (1756); "The Rout" (1758). As early as 1740 he wrote an opera, "Orpheus." *Cf.* D. E. Baker: "Biographia Dramatica," London, 1812 edition, pp. 341–348.

Stimulated, indeed, over-stimulated by this success, Dr. Hill now turned chiefly to his pen for his fortune and produced during the remainder of his life upward of eighty publications, not a few of which were in large folio. But such a statement gives no adequate idea of his prodigious capacity for work—"this gentleman may very justly be estimated as a phenomenon in literary history,—he was perhaps one of the most voluminous writers that this or any other age has produced." At one time he was engaged upon as many as seven volumes. Almost all branches of literature were included—fiction, the classics, theology, history and science; one or more volumes being devoted to astronomy, zoology, husbandry, botany, gardening, microscopy, medicine and the conduct of married life. In brief essays contributed daily for more than three years to the *London Advertiser,* under the pseudonym of *The Inspector,* there is hardly a phase of contemporary life or problems which he avoided. True, the inspector was primarily a monger in small

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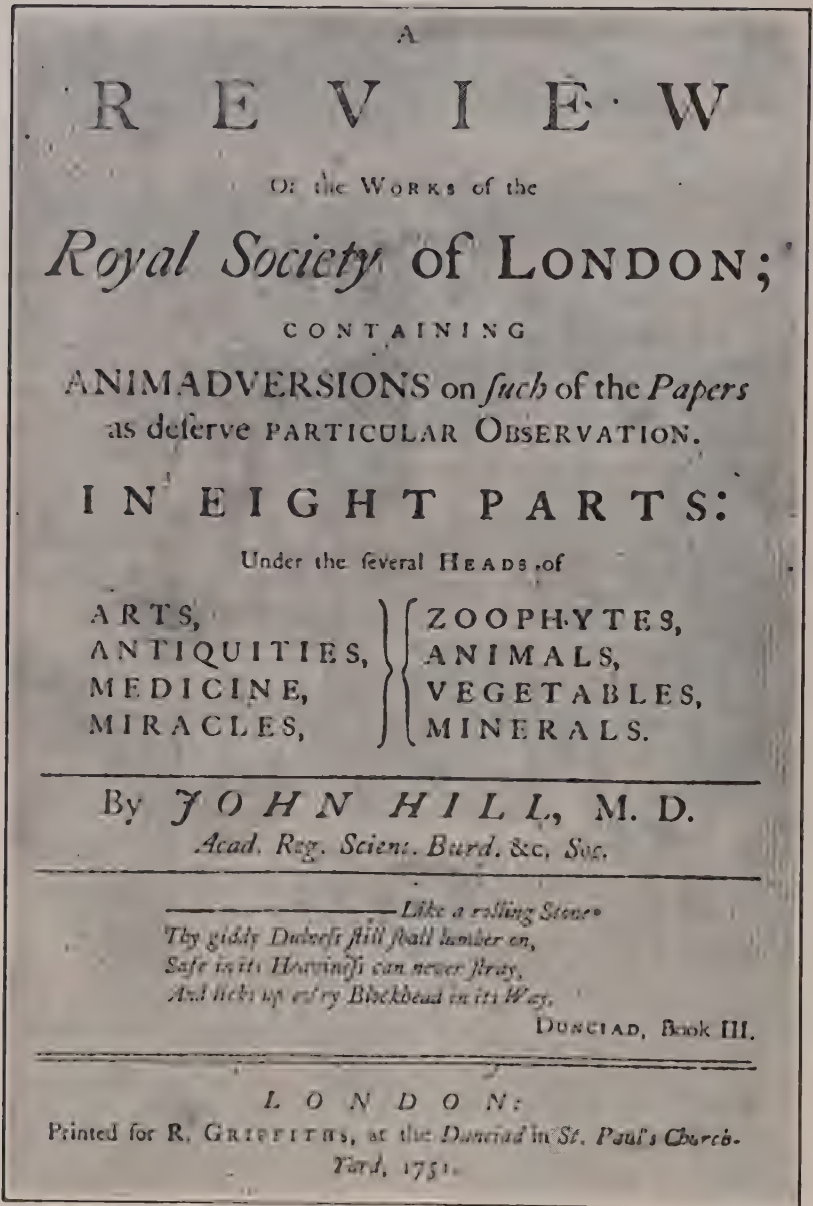
[4] L. L. Woodruff, "Baker on the Microscope and the Polype," *Scientific Monthly,* 7, 1918.



A

REVIEW

OF the WORKS of the

Royal Society of LONDON;

CONTAINING

ANIMADVERSIONS on *ſuch* of the *Papers*
as deſerve PARTICULAR OBSERVATION.

IN EIGHT PARTS:

Under the ſeveral HEADS of

ARTS,	ZOOPHYTES,
ANTIQUITIES,	ANIMALS,
MEDICINE,	VEGETABLES,
MIRACLES,	MINERALS.

By *JOHN HILL*, M. D.
Acad. Reg. Scient. Burd. &c. *Soc.*

——————Like a rolling Stone
Thy giddy Dulneſs ſtill ſhall lumber on,
Safe in its Heavineſs can never ſtray,
And licks up ev'ry Blockhead in its Way.
DUNCIAD, Book III.

L O N D O N:
Printed for R. GRIFFITHS, at the *Dunciad* in *St. Paul's Church-Yard*, 1751.

things, but this prototype of the columnist rises, now and again, distinctly above mediocrity in his daily output, especially when discussing natural history subjects.[5] Some of the Inspectors are of particular interest because they show that Hill was in many ways ahead of his times. One, for instance, suggests that botany would be greatly advanced by the delivery in the museum of public lectures illustrated by living plants—a foreshadowing of the laboratory method in biology; while another mentions the relationships of insects to pollination—a decade before Koelreuter. But, be their intrinsic value what it may, the daily grind was profitable to him in more than one way; it made him well known to the public, netted him a small fortune, and involved him in various quarrels with Garrick, Fielding, Smart and other literary men of the time—all of which was apparently to his liking.

It so happens that the contemporary estimate of Dr. Hill is chiefly preserved for us by the literary fraternity with whom he soon found himself at odds, and accordingly it is not surprising that his failings are not minimized and his virtues are largely overlooked. And the modern biographers of his associates accept unchallenged these estimates of the doctor-opponent of their paragons.[6] Nearly all find it necessary, for example, to recount at length the war of words between The Inspector and Fielding in his *Covent-Garden Journal*,[7] and to award the palm of victory to Fielding—after the lie had been passed—for his reply in which he refers to the doctor as "only a little paltry dunghill." Hill was not a match for the literary "giants" whom he inspected, though he was far from "losing his spirit in encountering the wits,"[8] and any one who will read without prejudice the best of his essays and compare them with the average output of his contemporaries can not fail to credit Hill with no mean literary ability.

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[5] "The Inspector." Reprinted with omissions and corrections, London, 1753.

[6] *Cf.* Frederick Lawrence, "Life of Henry Fielding," 1855; W. L. Cross, "The History of Henry Fielding," 1918.

[7] G. E. Jensen (editor), "Fielding's Covent-Garden Journal." Yale University Press, 1915.

[8] Isaac Disraeli, "Quarrels of Authors," London, 1814.

Samuel Johnson's fling at Dr. Hill took place, it will be recalled, when Johnson was reading in the King's Library and His Majesty, entering, began to interrogate him. Boswell gives it impressively:

> The King then asked him what he thought of Dr. Hill. Johnson answered, that he was an ingenious man, but had no veracity; and immediately mentioned, as an instance of it, an assertion of that writer, that he had seen objects magnified to a much greater degree by using three or four microscopes at a time than by using one. ''Now,'' added Johnson, ''everyone acquainted with microscopes knows, that the more of them he looks through the less the object will appear. . . .'' ''I now,'' said Johnson to his friends, when relating what had passed, ''began to consider that I was depreciating the man in the estimation of his Sovereign, and thought it was time for me to say something that was more favorable.'' He added, therefore, that ''Dr. Hill was, notwithstanding, a very curious observer; and if he would have been contented to tell the world no more than he knew, he might have been a very considerable man, and needed not to have recourse to such mean expedients to raise his reputation.''[9]

Whether Johnson's magnanimous final remarks, which amounted to reiterating that Hill was a liar, raised Hill in the estimation of his sovereign, does not appear, but the whole episode makes it clear that the King and Johnson were talking about something of which they knew nothing. Certainly ''if Hill's reputation for lying rests on no surer foundation than this, he must be acquitted of much that is charged to him.''[10] Of course, Dr. Hill was referring to lenses combined in the recently developed compound microscope. He himself had made certain minor improvements in the microscope which were embodied in an instrument offered for sale by the well-known London instrument maker, George Adams. Complete specifications with illustrations were given by Hill in his volume on ''The Construction of Timber'' (1770), and these were copied verbatim in the fourth edition (1771) of Adams's ''Micrographia Illustrata'' without so much as mentioning Hill. But this did not trouble Hill—he was well able to look out for himself. As one of the many epigrams which showered him put it:

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[9] James Boswell, ''Life of Samuel Johnson.''

[10] T. G. Hill, ''John Hill,'' in ''Makers of British Botany,'' F. W. Oliver, editor, 1913.

> Hill puffs himself; forbear to chide!
> An insect vile and mean
> Must first, he knows, be magnified
> Before it can be seen.[11]

Not content with arousing a storm among his literary contemporaries, Hill also raised consternation in the ranks of his scientific colleagues which reached its height when he made his famous attack on the Royal Society. It is not at all clear what gave rise to this onslaught since the various accounts do not agree. Hill attributed it to the perfidy of one of the fellows who, while calling at his house, took the liberty of reading a letter which Hill had written and left unsealed on his table. It appears, according to Hill, that a French correspondent, supposing him to be a fellow, had taxed him with "one of the errors of the Society," and Hill's letter stated, "I have already set right the error you complain of; but you are to know that I have the honor *not* to be a member of the Royal Society of London."[12]

Be this as it may, there is reason to believe that Hill became a candidate for election, since his publishers desired an F. R. S. after his name on their title pages, and failing of election because he had referred to various fellows as "cockelshell merchants," "medal scrapers" and "butterfly hunters," he launched a series of attacks including his indecent "Lucina sine concubitu" in 1750 and the following year his "Review of the Work of the Royal Society of London," both enjoying wide popularity in England and on the continent.[13] The latter appeared in the form of the *Philosophical Transactions,* and, after a rather lengthy preface, takes up about eighty papers published by the society. That animosity lurks in the author's mind is apparent from the dedication to his former patron, the president of the society, for Hill writes:

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[11] *Cf.* Disraeli, *loc. cit.*

[12] Preface, Hill's "Review."

[13] *Cf.* Hill's novel, "Adventures of Mr. George Edwards, a Creole," 1751.

> It is to you alone that the world owes their having been written; the purport of the more considerable of them [the criticisms] has been long since delivered to you in conversation; and if you had thought the Society deserved the censure that must attend this method of laying them before the world, you might have prevented it, by making the necessary use of them in private. Nor is this, Sir, the only sense in which you have been the great instrument of their production; since it cannot but be acknowledged, that if anybody, except your great self, had been in the high office you so worthily fill at present, the occasions of many of the more remarkable of them could not have been received by the body, under whose countenance alone they claim their places in this work.

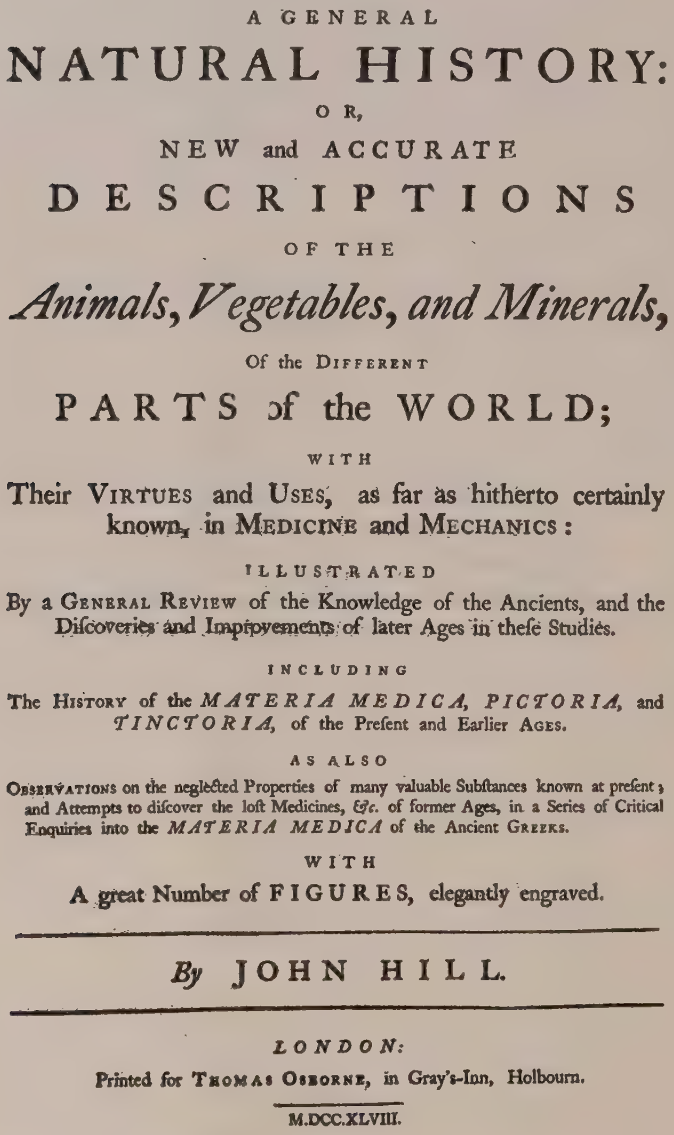
And Hill in the course of his preface modestly justified his method of treatment and his competence to criticize some of the learned fellows by stating that "if he is merry in some places, let it be considered, that the subjects are too ridiculous for serious criticism; if he is positive in others, let it not appear too assuming; he pretends to nothing but the knowing more than the Royal Society of London appears by its publication to know, and surely a man may do that, and yet be very ignorant!" However, he specifically states that "among the members of it, there are men great in all senses of the world, men esteemed in the highest degree by the author of these animadversions, and by whom he thinks it has greatest honor to be esteemed. . . These see too clearly to suppose a censure of the society under its present management, an attack upon their separate characters."

Two facts are clear from this Review. First, that Hill exhibits considerable ability as a literary opponent—his biting sarcasm and irony, frequently couched in happy phraseology, was never to be forgotten by the recipients of the thrusts. Second, that its author was one of the best informed scientific men of his day, who, from the standpoint of his actual scientific accomplishments, was far more worthy of honor than some of the fellows. And Hill knew it.

The opening paragraph of the Review is sufficient proof that the doctor had much fun—albeit a bit unfair—at the expense of the papers:

> A Way to Kill Rattlesnakes.—The ingenious author of the paper, in which this societarian method of destroying these poisonous and terrible animals is published, is Capt. Silas Taylor. It is indeed a kind of martial achievement, and worthy the title of the man who gives it; and as we dare venture to affirm that it will take place as well upon our own poisonous serpents, as on those of America, we could not omit giving it a conspicuous place, in a work intended like this of ours, for the general benefit of mankind. The method is delivered so early as in the third number of the *Philosophical Transactions*, and runs thus: Catch a rattlesnake, as large and vigorous as you please; fix it in any manner that you will, so that it cannot possibly get away; then procure a cleft stick, and put into the notch of it a quantity of the bruised leaves of wild pennyroyal; direct the end of the stick towards the serpent's nose; as he avoids it, still pursue him with it; and in half an hour's time he will be killed by the mere scent of the herb. . . . If the world should wish to see this amazing discovery parallelled by another more modern instance, we have a very late one in a countryman of our own, the ever to be remembered inventor of the powder for killing of fleas. The method of using this was very like that which the Captain here prescribes for his pennyroyal. The flea was to be held conveniently between the thumb and finger of the left-hand, while a very small quantity of the powder was applyed to the end of its trunk; after which, if the same flea could be proved ever to have bit the person again, he was to have another paper of the powder for nothing. How unhappy was it for the ingenious inventor of the method of destroying these vermin, that he did not apply himself to the Royal Society! The very first old woman he sold a paper to, unluckily asked him, whether, when she had got the flea, if she should crack it under her nail it would not be as well? The poor fellow could not but answer, that that way would do too, and was so thunderstruck with the objection that he never sold another. Such is the misfortune of a man's applying himself to old women, and being upon the spot, and in the way of having impertinent questions asked him: had he lived in New England or Virginia, and only communicated his discovery to a Royal Society, who of all its members would have thought of such a trifling objection to so useful a proposal?

And then Hill proceeds from paper to paper. One describes "a way to make all sorts of trees, plants, and fruits grow to an extraordinary bigness"—"sow the seeds or kernels, at the very instant when the sun enters into the vernal equinox," and "transplant them precisely at the moment when the moon is full"; another suggests "a way to make smelts grow to an extraordinary smallness''—''take the fish out of water which affords them a great deal of nourishment, and put them into such as affords them but a little,''[14] and so on. Although Hill asserts ''that the work might not be without its real use, an error is not here exposed without the establishing of



A GENERAL
NATURAL HISTORY:
OR,
NEW and ACCURATE
DESCRIPTIONS
OF THE
Animals, Vegetables, and Minerals,
Of the DIFFERENT
PARTS of the WORLD;
WITH
Their VIRTUES and USES, as far as hitherto certainly
known, in MEDICINE and MECHANICS:
ILLUSTRATED
By a GENERAL REVIEW of the Knowledge of the Ancients, and the
Diſcoveries and Improvements of later Ages in theſe Studies.
INCLUDING
The HISTORY of the *MATERIA MEDICA, PICTORIA,* and
TINCTORIA, of the Preſent and Earlier AGES.
AS ALSO
OBSERVATIONS on the neglected Properties of many valuable Subſtances known at preſent;
and Attempts to diſcover the loſt Medicines, *&c.* of former Ages, in a Series of Critical
Enquiries into the *MATERIA MEDICA* of the Ancient GREEKS.
WITH
A great Number of FIGURES, elegantly engraved.

By JOHN HILL.

LONDON:
Printed for THOMAS OSBORNE, in Gray's-Inn, Holbourn.
M.DCC.XLVIII.

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[14] ''Review,'' pp. 19–21.

the truth in the place of it,'' in a few cases he was himself in error, but this does not detract from the humor of the situation.

Clearly the society up to the time had not sufficiently profited by the *Tatler's* observation that ''their speculations do not so much tend to open and enlarge the mind, as to contract it and fix it on trifles,''[15] and the whole episode should have endeared Hill to Fielding and the literary fraternity in general, who enjoyed ridiculing the ''Experimentalists.'' It may be recalled that in the *Covent-Garden Journal,* Fielding refers to a very extraordinary woman ''who brought forth at one birth nineteen couple of rabbits, one of which having been eaten by the Royal Society and by them declared to have a most delicious relish, the breed was afterward propagated all over the Kingdom of England, were called Welsh Rabbits, and were a long time in great request.''[16]

So we find Hill during the fifties and sixties of the eighteenth-century a unique London character, with a considerable income from the voluminous publications which enabled him to keep himself before the public by driving about in his chariot, making himself conspicuous at coffee houses and theaters, and stirring up a paper war when that seemed necessary for notoriety. This would seem to be enough to consume the time of any one; but not so the doctor, for he was gardener at Kensington Palace, Justice of the Peace for Westminster, official adviser on horticulture to the governors of several tropical island colonies, and nominated, if not appointed, superintendent of the Royal Gardens at Kew. This exponent of the ''gentle art of making enemies'' was no ordinary man. A contemporary called him ''an extraordinary genius.''[17] Disraeli in his ''Quarrels of Authors'' says: ''Sir John Hill, this despised man, after all the fertile absurdities of his literary life, performed more for the improvement of the Philosophical Transactions, and was the cause of diffusing a more general taste for the science of botany, than any other contemporary.'' It is to Dr. Hill's actual contributions to biology that we now turn.

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[15] *Tatler,* No. 236.

[16] Jensen, *loc. cit.*

[17] Whiston, in Nichol's ''Literary Anecdotes of the Eighteenth Century,'' 1812.

We have already referred to Hill's first venture in science, his edition of ''Theophrastus on Gems.'' Its success led him to undertake the publication of a ''General Natural History'' in three large volumes between 1748 and 1752. Necessarily chiefly a compilation, it gave a ''Compleat History of the Three Great Classes of Natural Bodies'' which was second to none then extant in the English language. Von Haller recognized it as a work of infinite labor and great utility.

The volume devoted to fossils need not detain us since it offers no noteworthy original contribution, but shows clearly that the author knew his subject, and his interest in it persisted, because twenty-three years later he brought out one of the first manuals on mineralogy.[18] On the other hand, the volume on plants is of considerable importance because it introduced to English botanists the Linnaean system of plant classification.[19] This was two years before Linnaeus published his ''Species Plantarum,'' and accordingly was imperfect, but Hill incorporated the later advances in his ''Eden, or Complete Body of Gardening'' (1757); ''Gardener's New Kalender'' (1758); ''Flora Britannica'' (1760); and his ''Vegetable System'' (1759–1775). The preface of ''Eden'' declares ''The study of plants has undergone many variations with respect to method; and, at present, the system of Linnaeus is universally followed. No book on gardening has been written since this absolute change in the science. . . . Those who understand plants, now call them, universally and solely, by the names of this author. . . . We shall follow the system of Linnaeus, which we shall deliver completely, explaining his terms.''

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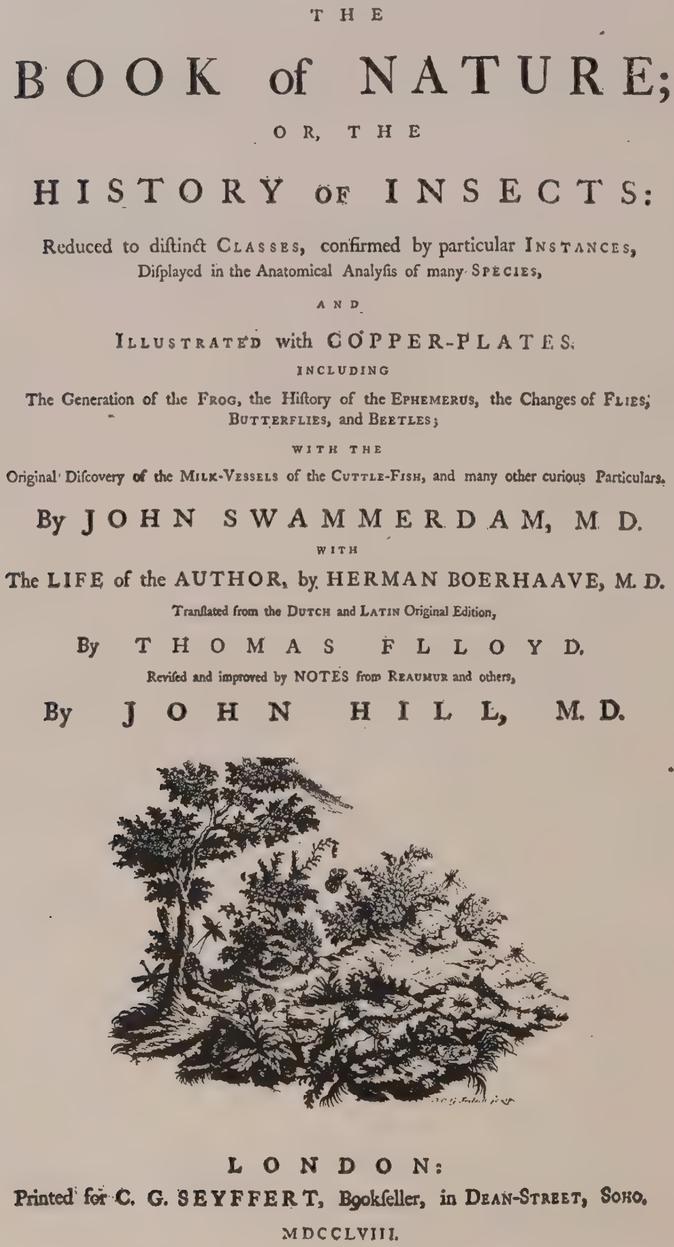
[18] ''Fossils Arranged According to their Obvious Character,'' etc., 1771.

[19] *Cf.* B. D. Jackson, ''Guide to the Literature of Botany,'' 1881.

The third volume of his "Natural History," devoted to animals, is noteworthy because it is the first work of the kind to include "accounts of the several classes of Animalcules, visible only by the assistance of microscopes." "Certainly their want of magnitude," the author points out, "does not exclude them from their rank among animated beings; yet this is the only History of Animals, in which they have been honored with a name. I have been at the pains of viewing all these by the microscope, and not according to the erroneous accounts of those superficial writers, who have seen others; but, from what appeared on that examination, I have arranged them into a regular method and given them denominations. The reader who is addicted to cavilling must not find fault with me for this. I have not changed their names, the greater part of them had none before." So Hill devotes Book I to Animalcules and attempts to classify them, coining many names which still persist—for example, *Paramecium.* It may well be doubted whether he did not overestimate his own critical study, if one can judge by certain of his figures of the Protozoa which obviously are copied from antiquated *Philosophical Transactions* of the Royal Society, and by some of his "Essays in Natural History and Philosophy," containing a "Series of Discoveries by the Assistance of Microscopes," which he published the same year. However, it can not be gainsaid that Hill was an expert with the microscope, probably second to none in England at the time, and his initial attempt to classify the Protozoa and other microscopic organisms was an important step in the right direction, which paved the way for intensive studies by other microscopists before the century closed.

Hill's other zoological work of importance is the English edition of Swammerdam's "Biblia Naturae." This monumental work of the great student of microscopic anatomy was published posthumously in Dutch and Latin under the editorship of Boerhaave in 1738, and fourteen years later appeared in a German translation, but it was not available in English until 1758, when Hill's edition was published. Hill did the work excellently, his notes



THE

BOOK of NATURE;

OR, THE

HISTORY OF INSECTS:

Reduced to diſtinct Classes, confirmed by particular Instances,
Diſplayed in the Anatomical Analyſis of many Species,
AND
Illustrated with COPPER-PLATES;
INCLUDING
The Generation of the Frog, the Hiſtory of the Ephemerus, the Changes of Flies,
Butterflies, and Beetles;
WITH THE
Original Diſcovery of the Milk-Vessels of the Cuttle-Fish, and many other curious Particulars.

By JOHN SWAMMERDAM, M. D.
WITH
The LIFE of the AUTHOR, by HERMAN BOERHAAVE, M. D.
Tranſlated from the Dutch and Latin Original Edition,
By THOMAS FLLOYD.
Reviſed and improved by NOTES from Reaumur and others,
By JOHN HILL, M. D.

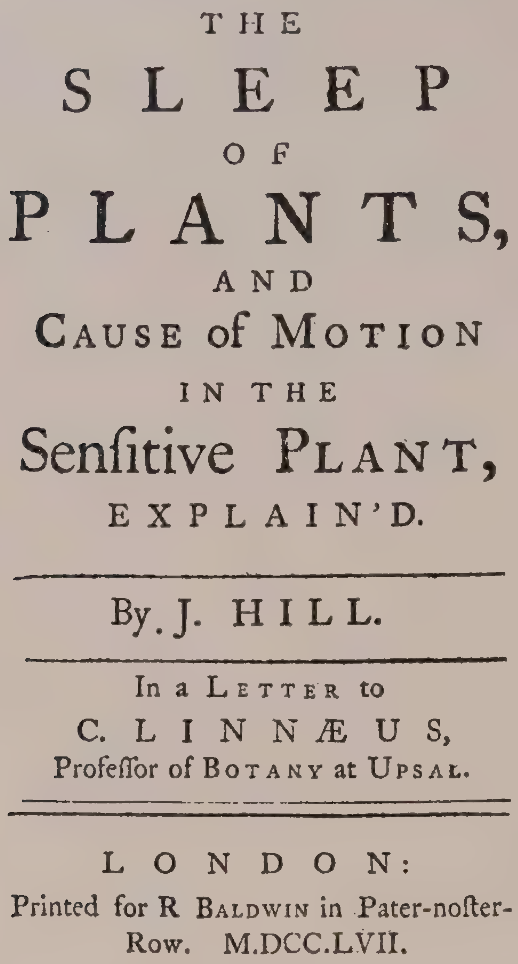
LONDON:
Printed for C. G. SEYFFERT, Bookſeller, in Dean-Street, Soho.
MDCCLVIII.

and comments add to its value, and the numerous plates of Swammerdam's remarkable dissections are well reproduced. Both Hill and his publishers deserve great credit for this standard edition of a biological classic.

In fairness to Hill's critics, mention should be made of his "Decade of Curious Insects," which appeared, richly illustrated in color, in 1773, two years before his death. Here he is at his worst, apparently giving his imagination free play, with no thought for his reputation and all for his pocketbook, though, if the truth be told, both were now at such a low ebb that it mattered little what he did.

But Hill was primarily interested in botany, and it is in this field that his scientific talents were chiefly displayed. His recognition of the importance of the Linnaean classification, which has already been mentioned, at once shows the breadth of his knowledge and the keenness of his foresight. Hill knew his plants and appreciated that the Linnaean system, with all its faults, which he does not hesitate to point out, offered a practical means of bringing order out of the existing chaos. Hill's commentators, who have found it impossible to reconcile his praise and criticism of Linnaeus's work, apparently overlooked this statement in Hill's "Sleep of Plants," published in the form of a letter addressed to the great botanist: "If our opinions have differed, 'tis upon a single point; your arrangement of plants. In regard to that much greater article, the establishing their distinctions, and ascertaining their characters, I have always admired and reverenced you: to dispute your determinations there, were to deny the characters of nature. Free in the tribute of applause on this head, I have on the other been as open in my censures; equally uninfluenced by envy, and by fear. It is thus science may be advanced; and you will permit me to say, thus men of candour should treat one another." Indeed, many of Hill's critics were by nature incapable of appreciating such sentiments.

Hill's chief works on systematic botany, in addition to those already mentioned, are the "British Herbal, an



THE
SLEEP
OF
PLANTS,
AND
CAUSE of MOTION
IN THE
Senſitive PLANT,
EXPLAIN'D.

By. J. HILL.

In a LETTER to
C. LINNÆUS,
Profeſſor of BOTANY at UPSAL.

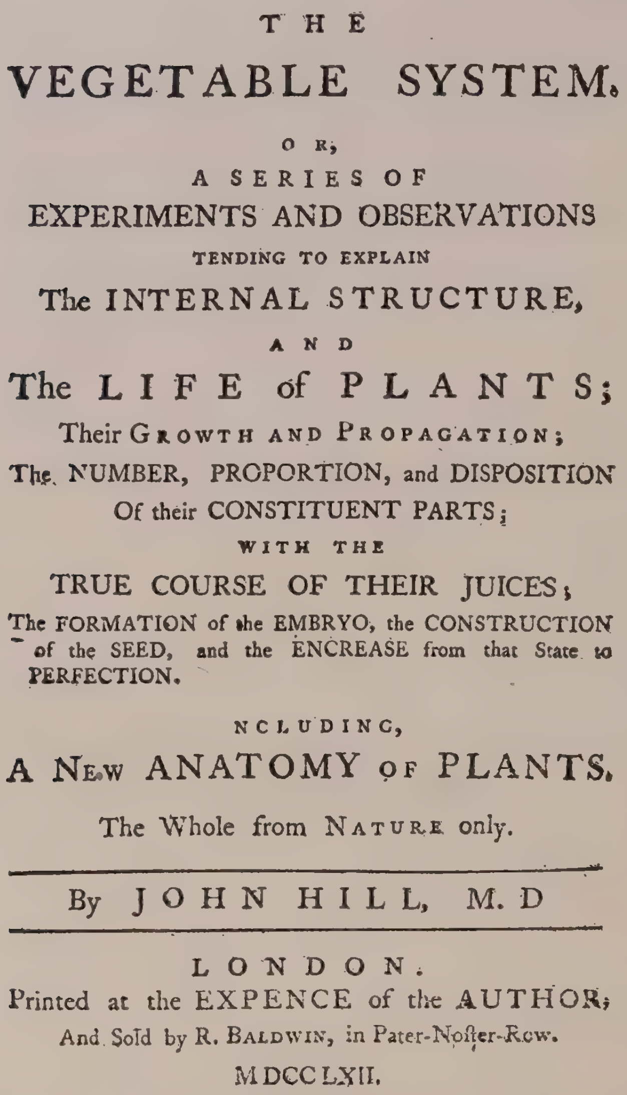
LONDON:
Printed for R BALDWIN in Pater-noſter-
Row. M.DCC.LVII.

History of Plants and Trees, Natives of Britain, Cultivated for Use or Raised for Beauty," a folio illustrated with more than seventy-five copper plates, and "The Vegetable System, or the Internal Structure and the Life of Plants," consisting of twenty-six folio volumes of text and plates.

"The British Herbal" was an outgrowth of his earlier "Useful Family Herbal," which, as its title page states, gave practical "directions for the gathering and preserving roots, herbs, flowers and seeds; the various methods of preserving these samples for present use; receipts for making distilled waters, conserves, syrups, electuaries, juleps, draughts, etc., with necessary cautions in giving them.'' But ''The British Herbal'' was a scholarly compendium for the botanist, gardener and apothecary in which Hill described and illustrated a very large number of plants with great accuracy, judged by the standards of botany in England at the time, and proved his ability in this field. ''The Vegetable System'' was started under the patronage of Lord Bute in 1759 and the twenty-sixth volume appeared in 1775, the year of Hill's death. The noble lord apparently gradually lost interest as the work progressed and the withdrawal of his financial support made the completion of the series with one thousand six hundred full-page plates a drain on the author's finances which they could not meet, though the price of the work to subscribers was 38 guineas plain and 160 guineas colored. The scope of the work may be judged by the fact that twenty-four volumes were devoted to a systematic description of British and foreign plants, and the remaining two chiefly to an outline of the history of botany, the systems of Cesalpino, Morison, Ray, Tournefort, Linnaeus and others, and to the development of plant morphology and physiology, including the effects of various physical factors on plant growth. A plate is devoted to his own experiments on *Abrus*.

Clearly, Hill had undertaken a project which was too great for one man, unless it was Hill himself, but he was turning out simultaneously volume after volume and pamphlet after pamphlet on other subjects to keep the wolf from the door. If this is borne in mind it is fair to say that it is a most remarkable work and a fitting culmination of Hill's studies in systematic botany.

But the doctor's methods of making herbals were not immune from gossip. Dr. Erasmus Darwin, the grandfather of Charles Darwin, in a letter written when a young man (1756), says: ''I believe I forgot to tell how Dr. Hill makes his 'Herbal.' He has got some wooden plates from some old herbal, and the man that cleans



THE
VEGETABLE SYSTEM.
OR,
A SERIES OF
EXPERIMENTS AND OBSERVATIONS
TENDING TO EXPLAIN
The INTERNAL STRUCTURE,
AND
The LIFE of PLANTS;
Their GROWTH AND PROPAGATION;
The NUMBER, PROPORTION, and DISPOSITION
Of their CONSTITUENT PARTS;
WITH THE
TRUE COURSE OF THEIR JUICES;
The FORMATION of the EMBRYO, the CONSTRUCTION
of the SEED, and the ENCREASE from that State to
PERFECTION.

NCLUDING,
A NEW ANATOMY OF PLANTS.

The Whole from NATURE only.

By JOHN HILL, M. D

LONDON.
Printed at the EXPENCE of the AUTHOR;
And Sold by R. BALDWIN, in Pater-Noſter-Row.
MDCCLXII.

them cuts out one branch of every one of them, or adds one branch or leaf to disguise them. This I have from my friend, . . . the watchmaker, to whom this printmender told it, adding, 'I make plants every day that God never dreamt of.' "[20] Suffice it to say that Hill, of course.

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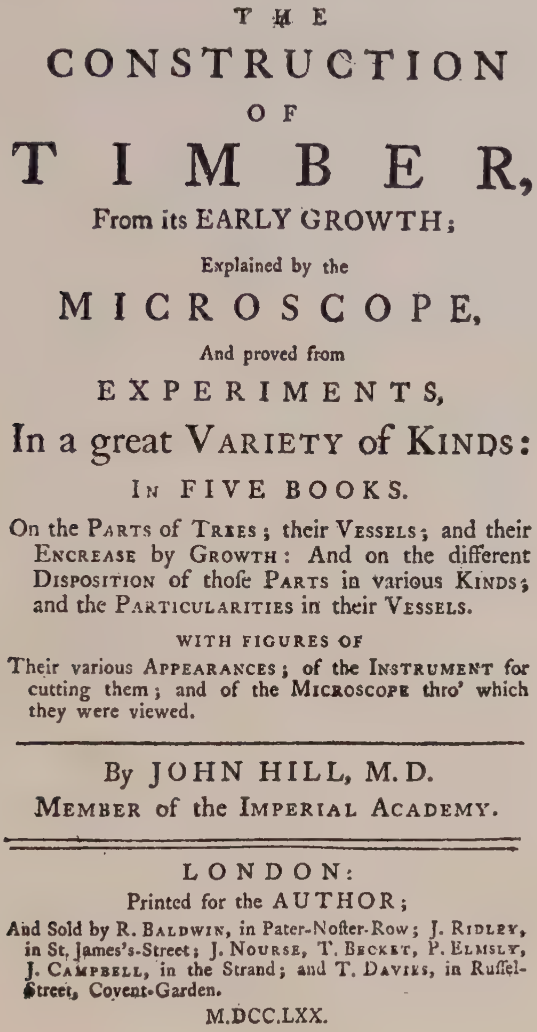
[20] Charles Darwin's introduction to E. Krause's "Life of Erasmus Darwin," p. 16.



THE
CONSTRUCTION
OF
TIMBER,
From its EARLY GROWTH;
Explained by the
MICROSCOPE,
And proved from
EXPERIMENTS,
In a great VARIETY of KINDS:
IN FIVE BOOKS.
On the PARTS of TREES; their VESSELS; and their ENCREASE by GROWTH: And on the different DISPOSITION of thoſe PARTS in various KINDS; and the PARTICULARITIES in their VESSELS.
WITH FIGURES OF
Their various APPEARANCES; of the INSTRUMENT for cutting them; and of the MICROSCOPE thro' which they were viewed.

By JOHN HILL, M.D.
MEMBER of the IMPERIAL ACADEMY.

LONDON:
Printed for the AUTHOR;
And Sold by R. BALDWIN, in Pater-Noſter-Row; J. RIDLEY, in St. James's-Street; J. NOURSE, T. BECKET, P. ELMSLY, J. CAMPBELL, in the Strand; and T. DAVIES, in Ruſſel-Street, Covent-Garden.
M.DCC.LXX.

drew upon his *own* early herbals for text figures when writing his later ones. Hill's position as a systematic botanist is attested by Jacquin naming in his honor a genus, *Hillia,* and by the number of species in our flora to-day which are credited to him, from the establishment of the year 1753 as the base line for plant nomenclature. The King of Sweden recognized the value of the "Vegetable System" by granting its author the Order of Vasa, and thereafter Hill employed the title, Sir. He was also elected a member of the Imperial Academy and a fellow of the Royal Academy of Sciences at Bordeaux.

Systematic botany was in vogue when Hill was writing, so it is not strange that he devoted himself largely to this aspect of the science, but it is rather remarkable that he undertook some interesting studies in plant anatomy since, during the eighteenth century, little had been added to the basic work of Malpighi and Grew.

Hill's investigations in this field are included in a volume entitled "The Construction of Timber, from its Early Growth; Explained by the Microscope and proved from Experiments in a Great Variety of Kinds." This was published in 1770 and a new edition appeared four years later with an additional chapter on "the strange disposition of the vessels in the ferns." The work shows that Hill possessed unusual technical ability with a newly invented instrument for section cutting, the forerunner of the modern microtome, and with his own improved compound microscope. After describing these devices, the author proceeds to a general account of tissues and their distribution in plant stems, taking up his observations under five chief headings: "the constituent parts of timber; the vessels of trees; the encrease by growth; the different disposition of the parts in various trees"; and "the particularities in the vessels of trees." The critical comparison of the tissues in various plants is well illustrated with copper plates which represent sections not very highly magnified, but nevertheless bear favorable comparison with many in the works of Grew and Malpighi. Hill's incursion into plant histology, prompted by a life-long interest in studies with the microscope, foreshadows the intensive work by other investigators as the century closed. Hill, however, left the subject almost where he found it.

As early as 1757 Hill became interested in the physiology of plants and published a pamphlet, already mentioned, entitled "The Sleep of Plants and Causes of Motion in the Sensitive Plant Explain'd,'' in the form of a letter addressed to Linnaeus, who had referred to the phenomenon in question as ''sleep.'' The same year the observations were incorporated in his large volume, ''Eden, or a Compleat Body of Gardening.''[21]

A critical study of the monograph shows clearly that Hill possessed unusual aptitude for the analysis of physiological phenomena by the experimental method, a talent by no means commonly exhibited at the time, although Hales's ''Vegetable Staticks'' had shown the way. For example, Hill emphasizes the necessity of studying separately the various physical factors of the plant's environment and then combining them in the ways they normally act upon the organism; the importance of keeping all the conditions, except the one purposely varied, absolutely constant during experimentation, and, finally, the value of comparing the results from experiments on different species of plants. Thus, in studying the response to light of *Mimosa* and *Abrus,* he found that ''in these and in all others, the degree of elevation or expansion in the lobes is exactly proportional to the quality of the light: and is solely dependent upon it.'' Hill, of course, made mistakes in his observations and interpretations, but taken as a whole this brief study is a neatly executed and most interesting contribution, antedating by a year Du Hamel's well-known ''Physique des arbres.''

Turning from the ''Sleep of Plants,'' Hill found time to publish in 1758 a monograph entitled ''Outlines of a System of Vegetable Generation,'' in which he endeavored to show that the pollen grain contains an embryo which it sets free when deposited on the stigma. Obviously, he saw the contents of the pollen grain and misinterpreted it as an embryo. He noted that pollen grains burst when placed in a drop of water and concluded that a similar bursting and setting free of the embryo took place on the wet stigma, a phenomenon rediscovered 138 years later by Lindforss.

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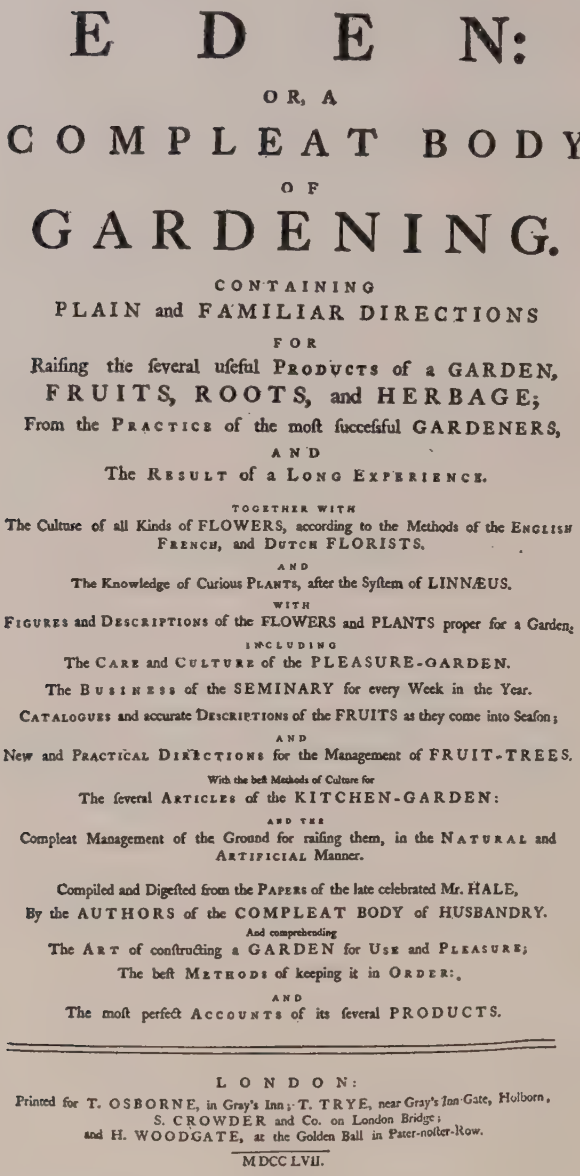
[21] Pp. 703–708.



EDEN:
OR, A
COMPLEAT BODY
OF
GARDENING.
CONTAINING
PLAIN and FAMILIAR DIRECTIONS
FOR
Raiſing the ſeveral uſeful Products of a GARDEN,
FRUITS, ROOTS, and HERBAGE;
From the Practice of the moſt ſucceſsful GARDENERS,
AND
The Result of a Long Experience.
TOGETHER WITH
The Culture of all Kinds of FLOWERS, according to the Methods of the English
French, and Dutch FLORISTS.
AND
The Knowledge of Curious Plants, after the Syſtem of LINNÆUS.
WITH
Figures and Descriptions of the FLOWERS and PLANTS proper for a Garden,
INCLUDING
The Care and Culture of the PLEASURE-GARDEN.
The Business of the SEMINARY for every Week in the Year.
Catalogues and accurate Descriptions of the FRUITS as they come into Seaſon;
AND
New and Practical Directions for the Management of FRUIT-TREES.
With the beſt Methods of Culture for
The ſeveral Articles of the KITCHEN-GARDEN:
AND THE
Compleat Management of the Ground for raiſing them, in the Natural and
Artificial Manner.

Compiled and Digeſted from the Papers of the late celebrated Mr. HALE,
By the AUTHORS of the COMPLEAT BODY of HUSBANDRY.
And comprehending
The Art of conſtructing a GARDEN for Use and Pleasure;
The beſt Methods of keeping it in Order:
AND
The moſt perfect Accounts of its ſeveral PRODUCTS.

LONDON:
Printed for T. OSBORNE, in Gray's Inn; T. TRYE, near Gray's Inn Gate, Holborn,
S. CROWDER and Co. on London Bridge;
and H. WOODGATE, at the Golden Ball in Pater-noſter-Row.
MDCCLVII.

Even a brief survey of the "Proteus Hill," the "Cain of Literature," the "quack and blustering adventurer," the "Halloway of his day," the man with "cowardice that seemed a disease"—to borrow epithets from biographers of his enemies—would be incomplete without at least mentioning by title a few other samples of his prodigious literary output: "A History of the Materia Medica" (1751), "Adventures of Mr. George Edwards, a Creole" (1751); "The Story of Elizabeth Canning Considered. With remarks on what has been called A Clear State of her Case, by Mr. Fielding" (1753); "Observations on the Greek and Roman Classics: Series of Letters to a Young Nobleman, now published for the Use of Gentlemen at the University" (1753); "Urania, or a Compleat View of the Heavens; Containing the Ancient and Modern Astronomy in Form of a Dictionary" (1754); "Thoughts Concerning God and Nature, in Answer to Lord Bolingbroke's Philosophy" (1755); "The Maiden's Whim, or the Critical Minute"—a play (1756); "The Naval History of Britain" (1756); "Compleat Body of Husbandry" (1756); and "The Management of the Gout, with the Virtues of Burdock Root" (1758).

Such are some other titles which attract our attention during merely the first decade of his career. Much, to be sure, is hack work, but most of it is exceedingly well done. His "Husbandry" and "Gardening" are among the first works in which scientific knowledge was put in a popular form by being issued in weekly installments. He was still "going strong" to the day of his death, writing pamphlets on the virtues of sage, polypody, bardana and the rest which enjoyed a wide sale and gave him a living, while he was struggling to publish the last volumes of the "Vegetable System." That he believed in the efficacy of the herbs he exploited there is little reason to doubt. More than once he expressed his view that the modern "chemical medicines" were dangerous, especially when not specifically prescribed by a physician. Perhaps a great deal can be read between these lines from his "Virtues of British Herbs": "He who seeks the herb for the cure, will find it half effected by the walk." This aspect of the doctor was crystallized in many a literary compliment:

Thou essence of dock, valerian and sage,
At once the disgrace and the pest of the age;
The worst we can wish you for all your dam'd crimes
Is to take thy own physics and read thy own rhymes.[22]

And, as if this were not enough, the famous "Mrs. Glasse's Cookery" was attributed to Hill. Surely we must credit him with moral courage in enduring "with undiminished spirit the most biting satires, the most wounding epigrams, and most palpable castigations." To Smart's "Hilliad," which called him "Th' insolvent tenant of encumber'd space," he flung a "Smartiad";[23] and to the charge of unbounded conceit and effrontery, he replied, "No one knows more the narrow limits of human knowledge; or entertains an humbler opinion of the returns of years of application."

Still another facet of Hill's many-sided personality is revealed by his stout volume, "Thoughts Concerning God and Nature, in Answer to Lord Bolingbroke's Philosophy," which reveals a breadth of view and familiarity with a field of literature which might not be expected of the doctor. The volume was apparently published at some expense to the author, since he says that if the entire edition were sold, it would not cover the cost of printing. The book is in no sense a stereotyped "natural theology" after the fashion of the time, though he justly states: "It is certain that God is to be perceived by the most slight view of the universe; and it is as certain, that the more precise and accurate that view is made, the more plainly, the more fully and the more distinctly that God is seen. This is the last affirmation of all true wisdom."

A summary view of Dr. John Hill—indeed, any view must be a summary—though from the vantage point of

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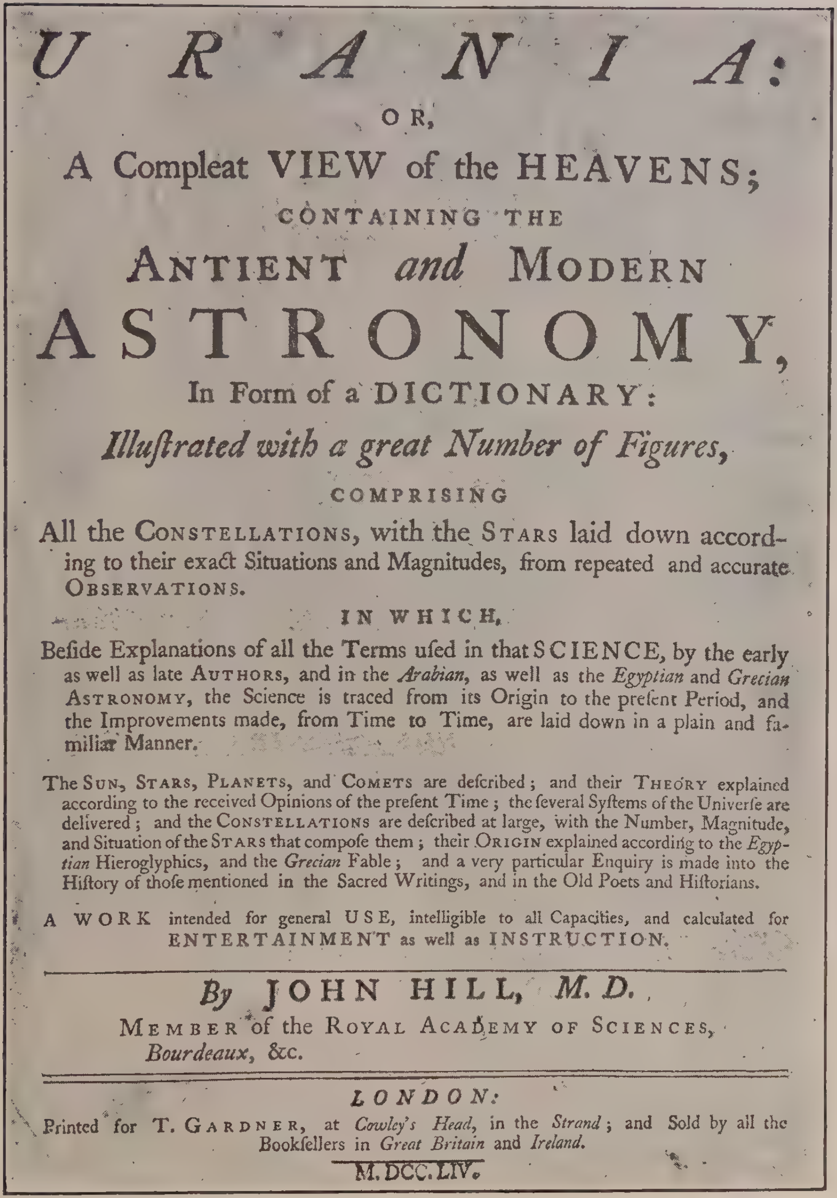
[22] *Cf.* Percy Fitzgerald, "Life of David Garrick," Rev. Ed., 1899.

[23] Christopher Smart, "The Hilliad," London, 1753. Republished in Reed's Repository, London, 1783. John Hill, "The Smartiad," London, 1753.



URANIA:

OR,

A Compleat VIEW of the HEAVENS;

CONTAINING THE

ANTIENT *and* MODERN

ASTRONOMY,

In Form of a DICTIONARY:

Illuſtrated with a great Number of Figures,

COMPRISING

All the CONSTELLATIONS, with the STARS laid down according to their exact Situations and Magnitudes, from repeated and accurate OBSERVATIONS.

IN WHICH,

Beſide Explanations of all the Terms uſed in that SCIENCE, by the early as well as late AUTHORS, and in the *Arabian*, as well as the *Egyptian* and *Grecian* ASTRONOMY, the Science is traced from its Origin to the preſent Period, and the Improvements made, from Time to Time, are laid down in a plain and familiar Manner.

The SUN, STARS, PLANETS, and COMETS are deſcribed; and their THEORY explained according to the received Opinions of the preſent Time; the ſeveral Syſtems of the Univerſe are delivered; and the CONSTELLATIONS are deſcribed at large, with the Number, Magnitude, and Situation of the STARS that compoſe them; their ORIGIN explained according to the *Egyptian* Hieroglyphics, and the *Grecian* Fable; and a very particular Enquiry is made into the Hiſtory of thoſe mentioned in the Sacred Writings, and in the Old Poets and Hiſtorians.

A WORK intended for general USE, intelligible to all Capacities, and calculated for ENTERTAINMENT as well as INSTRUCTION.

By JOHN HILL, *M.D.*

MEMBER of the ROYAL ACADEMY OF SCIENCES,
Bourdeaux, &c.

LONDON:
Printed for T. GARDNER, at *Cowley's Head*, in the *Strand*; and Sold by all the Bookſellers in *Great Britain* and *Ireland*.
M.DCC.LIV.

nearly two elapsed centuries, does neither Hill nor his enemies justice. So much, however, is clear: Hill was endowed with remarkable talents which warranted his holding a commanding position among scientific men, but he largely dissipated this birthright by a temperament which made himself his own worst enemy. A contemporary, who was not a friend, said the doctor was "of all men I ever knew so mixed a character, none but himself can be his parallel." And this estimate can not be improved to-day.

Reprinted from the Transactions of the Twenty-second Annual Meeting
of the National Tuberculosis Association
1926

THE ORIGIN OF THE FIBROUS TISSUE ARISING IN THE GUINEA PIG TESTIS AS A RESULT OF EXPERIMENTAL TUBERCULOSIS* †

By George A. Baitsell, Ph. D., and Karl E. Mason, Ph. D.

New Haven, Conn.

INTRODUCTION

It has long been recognized that one of the prominent histological features of tissues infected with tubercle bacilli is the formation of a comparatively large amount of fibrous tissue which infiltrates and encapsulates the tubercle and, in general, appears in abundance throughout the infected tissues. As to the method of formation of the fibrous tissue arising under such conditions and as to whether it aids in the control of the disease or aids in its further dissemination, conclusive evidence appears to be lacking at present. It was with the hope of supplying additional information regarding these points that the present studies were undertaken. The answer to all the points involved will lead necessarily to a consideration of the entire history of the infected tissues from the first entrance of the bacilli until, on one hand, they are under complete control, or on the other hand, they have destroyed the infected tissues. The data so far available, as presented in the present communication, are limited to the question of the origin of the fibrous tissue arising in the testes of guinea pigs following experimental infection, and leave untouched other questions involving the cellular origin of the tubercle as well as the effect, if any, of the fibrous tissue on the life history of tubercle bacilli. The results presented in this paper show that the fibrous tissue in the infected guinea pig testis arises by a transformation of an intercellular exudate independently of any direct cellular connection.

MATERIALS AND METHODS

The selection of the testicular tissues as a field for the study of the origin of the fibrous tissue was due to a number of factors. Long[1] has recently shown that the testicular tissues are very sensitive to tubercle bacilli and to tuberculin. In the latter case the reaction of the testicular tissue, as shown by the amount of degeneration, is used as a direct indicator of the strength of the tuberculin solution. The histological study of testicular tissues following the injection of tuberculin shows that there is a rapid and complete degeneration of the germinal cells of the seminiferous tubules followed by their elimination from the testis. This response of the testis to tuberculin is not a specific reaction, but as has been shown by various authors[2], may result from other factors such as diet deficiency, temperature changes etc. The immediate response of the testicular tissues to the injection of living tubercle bacilli, as shown by Long *(op. cit.)* appears to be the same, in general, as in the case of the tuberculin injection. Later, however, when bacilli are injected, a progressive degeneration of the tubules themselves may occur together with a heavy cellular infiltration and later a prolific formation of fibrous tissue. The fibrous tissue apparently does not form in the testis except in the case of the injection of the living bacilli.

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* From the Osborn Zoölogical Laboratory, Yale University.

† Work done under a grant from the Medical Research Committee of the National Tuberculosis Association.

One of us (Mason[3]) has shown that in the degenerating germinal cells of the rat, due to the cumulative effects of a diet deficient for reproduction, mulinucleate giant cells are frequently found in the tubules. These have been formed by a fusion of the germinal cells. It was thought that this process of giant cell formation in the tubules by a direct fusion of cells might throw light on the process of giant cell formation in the tubercles which would be expected to develop later in the infected intertubular areas. It would be an interesting fact if it were found that giant cells were capable of being formed in the testis either by cellular fusion within the seminiferous tubules or by the rapid growth and repeated division of a single cell in the intertubular areas.

The exposed position of the testes together with their paired condition offer additional advantages for use in this work. The tubercle bacilli can, with little difficulty, be injected into any desired region of one or both testes. Finally, the unique histological structure of the testis offers great advantages in interpreting the changes which occur following the infection.

In the present experiments, pairs of male guinea pigs of approximately equal weight were chosen. One of each pair was given a sensitizing inoculation, either axillary or intraperitoneal, of tubercle bacilli of low virulence (R 1 Saranac) three weeks previous to a direct testicular inoculation in which highly virulent tubercle bacilli were used. The testicular inoculation was given in only one testis; the other member of each pair of testes being used for comparison. These animals which have received the double infection are referred to below as "reinfected animals." The other guinea pig of each pair received only the testicular inoculation, nd these animals are referred to as “control animals.” The animals were killed by chloroform at definite intervals, a careful autopsy made of all the organs, and specimens of the various tissues preserved for histological studies.

STUDY OF THE PREPARED MATERIAL

The study of permanent preparations of guinea pig testes, which have been infected with tubercle bacilli, shows a consistent and striking series of changes which result in the radical transformation of the normal histological picture of the testis. The changes occur with much greater rapidity and, in the early stages, are more pronounced in the reinfected animals than in the control animals which have received only the direct esticular inoculation. In the later stages, however, the histological picture of the testis is, in general, the same in both types of experimental animals.

The testicular changes induced by the experimental infection are marked by (1) the degeneration and elimination of the germinal cells from he seminiferous tubules; (2) the presence of a large amount of exudate nd a cellular infiltration in the intertubular areas; and (3) the formation of a large amount of fibrous tissue. We may now proceed to discuss briefly, each of these changes.

(1) Following the testicular inoculation there is a rapid degeneration nd elimination of the germ cells, involving extensive sloughing, pycnosis, necrosis, and formation of multinucleate giant cells through the fusion of adjoining germ cells. In the testes of reinfected animals these changes begin very quickly following the testicular inoculation, and within a few hours are quite noticeable. In the testes of control animals the process s much slower and some days will elapse before there is a marked departure from the normal condition. In both cases most of the degenerating germ cells are passed out of the tubules, leaving the latter greatly reduced in size, and consisting only, in many instances, of Sertoli cell nuclei imbedded in a fibrous-appearing material.

(2) In the testes of reinfected animals, a great amount of exudate s found in the intertubular areas shortly after the testicular inoculation. Thus in preparations of testis made from material taken two days after the testicular inoculation, it will be found that practically all the intertubular areas are not only filled with the exudate, but as a rule, are considerably enlarged due apparently to the great abundance of this material which has been poured in. The exudate appears much more slowly in the testis of a control animal. In the latter case, preparations made from testicular tissues taken six days after infection how an early stage in the degenerative processes. Many of the in-

tertubular areas are practically free from exudate just as in a normal testis, while in other regions small areas of exudate may be noted. The exudate may contain considerable numbers of infiltrating cells, or in certain regions it may be almost free of cells.

The exudate which arises in the intertubular areas appears, when studied in the early stages, to be almost homogeneous in structure. Close observation, however, with high magnification will generally show a fine fibrillation to be present, and occasionally an area may be found in which the fibrillation of the exudate is quite pronounced. It is very clear from a study of preparations taken at longer periods after the infection that the fibrillation of the exudate shows a progressive increase in the older stages. Varying degrees of cellular infiltration occur in the intertubular areas along with the exudate. There is considerable evidence from the preparations to show that the exudate is present in the intertubular areas previous to the cellular infiltration. Presumably the exudate is utilized as a supporting material by the inwandering cells which move through it.

(3) We turn now to the question with which this communication is primarily concerned, namely the origin of the fibrous tissue in the infected testes. It is formed in such great abundance that, in the present experiments, within four weeks after the testicular infection, in both the reinfected and control animals, the testis has become largely transformed into a mass of fibrous tissue. Results obtained from a careful study of large numbers of permanent preparations of infected testicular tissues in various stages of degeneration give a consistent and striking picture of the formation of the fibrous tissue by a direct transformation of the exudate.

The process begins as a fine fibrillation as noted in the early stages, later appears as a reticulum, and finally reaches its climax in the formation of heavy bundles of wavy fibers which take a beautiful Mallory stain and which encapsulate and infiltrate the tubercle as well as the affected tissues in general. Throughout the various stages in the transformation of the exudate into the typical fibrous tissue the evidence from the study thus far made shows that the process is not dependent upon a direct cellular connection. In other words, the process is fundamentally intercellular and not intracellular. This does not preclude the possibility that the movements of the cells through the exudate may not by means of purely mechanical factors change the structure of the fibrous material, or that the secretions of various types of cells may so influence the fibers that their chemical composition becomes changed. The method of formation of the fibrous tissue appears, therefore, to be fundamentally of the same type as has been shown to occur in tissue cultures[4], in wound healing[5], and in embryonic development[6].

In the complete paper, which it is expected will be published at an arly date, figures showing progressive stages in the transformation of e exudate will be given together with a discussion of the points involved.

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3208

Disintegrative Action of KNC on Amblystoma Embryos in Solutions of Different Osmotic Pressures.

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A series of experiments were performed dealing with the effect of varying the osmotic pressure of the solution, on the disintegrative action of potassium cyanide, on the early embryos of *Amblystoma punctatum*. The experiment was carried on during the Spring of 1926. Owing to the unusually short season, the work is incomplete in many respects, and further experiments are planned for future seasons. However, certain very definite results have been obtained, which appear to have an important bearing on the general problem of susceptibility and susceptibility gradients.

In each experiment a batch of embryos from a single female was selected and dissected free from the jelly capsules. This was necessary because preliminary experiments showed that the capsules afford some protection against KNC. The embryos were allowed to remain for a number of hours in cold running tap water, during which time any that had been injured died. To freshly made solutions of commercial cane sugar, Ringer's solution, urea (made up in both distilled and tap water), and to distilled and tap water was added sufficient KNC to make the desired concentration. The range of concentrations employed was: Sugar, 0.03 molal to 0.6 molal; Ringer's solution, 1/10th to double the usual concentration; urea, 0.03 molal to 0.6 molal; KNC M/250 to M/125. As regards the osmotic pressure of the solutions, a crude cyroscopic method served to demonstrate that solutions of sugar of the order of 0.15 molal and Ringer's solution diluted one-half are both hypertonic for the Amblystoma embryo in the stages employed. Saturation with oxygen was assured by shaking the solutions with air. The embryos were then placed in 250 cc. Stender dishes in tap water, and the water replaced by 250 cc. of the desired solutions. The lids of the dishes were sealed with vaseline. Examination of the condition of the embryos was made at suitable intervals, and a record was made of all that showed any indications of death or disintegration. Depending on the number of embryos in the batch, groups of ten or twenty were placed in each dish. The stages used were blastulae and gastrulae. No attempt was made to bring the solutions to neutrality, hence, the results are the sum of the specific effect of the reagents and that of the alkalinity of the solutions. The distilled water used was once distilled in a copper still equipped with a block tin condenser.

From the data obtained in 15 experiments thus far completed, the following facts are clear: (1) Embryos die more rapidly in distilled water solutions of KNC than in like concentrations made up in tap water. The difference is equally evident between distilled and tap water solutions of urea, hypertonic sugar, and hypertonic Ringer's, to which KNC has been added. The additive action of KNC and distilled water of the sort employed here is, therefore, plainly not due to the hypotonicity of the KNC-distilled water solutions.

(2) In general, embryos disintegrate more rapidly in solutions of cyanide made up in Ringer's solution in at least two concentrations (full strength and one-half strength) than in such solutions made up in tap water. This additive action of KNC and Ringer's solution makes it necessary to observe caution in drawing conclusions regarding the rôle of osmotic pressure in the disintegrative action of KNC from results obtained with Ringer's-cyanide solutions.

(3) Urea is toxic in solutions iso-molal with non-toxic hypertonic concentrations of sugar. Conclusions regarding the effect of hypertonic solutions on development as compared with the effect of iso-molal solutions of urea are, therefore, of limited value.

(4) Cane sugar exerts an antagonism toward the disinte-

grative action of KNC. The protection afforded by the sugar increases with increasing concentration of the sugar up to 0.3 molal. There were no exceptions observed. The results indicate that with increasing concentration of the cyanide, the concentration of sugar necessary to afford greatest protection increases.

Embryos which were intact in strong sugar-cyanide solutions at the end of 48 and 72 hours were transferred to the same concentration of sugar without the cyanide, and the concentration of sugar decreased gradually by the addition of tap water. Some of the embryos disintegrated very soon, others developed slowly for three or four days and then died. From this it may be concluded that while sugar protects the embryo against the disintegrative action of the cyanide, nevertheless the injury is profound and complete recovery does not occur after the exposures employed here.

Indications of death of the embryos differ among the solutions used. In one-half Ringer's solution plus M/250 KNC death in blastulae begins as a gray cap at the animal pole. This cap increases in area and spreads downward and eventually involves the entire surface. Blastulae do not usually burst during the first 72 hours of exposure. The process is similar but much more rapid in 0.15 molal urea plus KNC, and in tap and distilled water solutions of KNC, and is frequently accompanied by the bursting of the blastulae in the vegetal hemisphere. In the case of gastrulae, the region of the closing blastopore is first affected in these solutions. Visible changes are the whitening of the cells about the blastopore, particularly the dorsal lip, followed by the spreading of the whitened area anteriorly involving the neural plate. The neural plate may persist for some time as an area of dead tissue before other regions of the embryo are visibly affected.

Deaths occurring in hypertonic sugar-KNC solutions do not show this differential susceptibility of the animal pole, blastoporic lips, and neural plate. As stated above, death and disintegration in hypertonic sugar-KNC solutions is greatly delayed as compared with the rapidity of these processes in KNC added to the other agents employed. When disintegration finally does occur, no differences in the appearance of the animal pole of blastulae, nor the lips of the blastopore and neural plate of gastrulae, and other regions of the embryo are discernible. There were no exceptions to this among the more than 50 embryos dying in sugar-KNC solutions that were observed. The entire embryo becomes a lighter color and eventually bursts in the region of the yolk cells. In 0.6 molal sugar plus KNC whitened ridges appear, probably due to shrinkage in this concentration of sugar. The processes of disintegration begin at these ridges and soon spread and involve the entire embryo.

Two main conclusions may be drawn from the results of the experiments with sugar-cyanide solutions; first, as regards the disintegration of Amblystoma embryos, KNC and hypertonic sugar solutions are antagonistic. This fact is in line with the well known experiments of Loeb, who employed KNC to prevent the disintegrative action of hypertonic sea water on sea urchin eggs. Second, hypertonic sugar solutions obliterate the differential susceptibility of the several regions of the embryo to cyanide disintegration. Observations on a possible similar action of strongly hypertonic Ringer's solution are incomplete.

THE INFLUENCE OF VOLUME OF CULTURE MEDIUM AND CELL PROXIMITY ON THE RATE OF REPRODUCTION OF INFUSORIA

A DISSERTATION
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CONTENTS

Introduction 143
Materials and methods 148
Experiments 149
  A. The influence of volume of culture medium on the rate of reproduction 150
  B. The effect of cell proximity on the rate of reproduction 153
  C. Repressed division rate 155
    1. Animals transferred from old mass cultures 155
    2. Animals transferred from young mass cultures 158
  D. The division rate of Infusoria bred forty-eight hours in the same medium, compared with the division rate of sister cells changed daily 162
Conclusions 164
Summary 166
Literature cited 167

INTRODUCTION

The influence of such factors as volume of culture medium, cell proximity, and age of culture medium upon the division rate of Infusoria has been variously interpreted.

Woodruff(8), in 1911, showed that the division rate of Paramecium aurelia and Paramecium caudatum was more rapid in larger volumes of culture medium within certain limits. The animals were carried in volumes of two, five, twenty, and forty drops of culture medium and changed every twenty-four hours to culture medium made up fresh every forty-eight hours, with the result that the division rate of the animals in the higher volumes was found to be more rapid. He also considered the governing factor or factors. Temperature was eliminated from a place of importance, since it was the same for all of the experiments conducted at the same time. The pressure variations in the several volumes were regarded as being so exceedingly slight as to warrant the belief, since there was no proof to the contrary, that this was not a governing factor. The surface exposure, especially in the larger volumes, permitting a more rapid exchange of gases was recognized as being of probable importance, so that every effort was made to counterbalance this factor by the use of ''receptacles of different capacities and shapes for the different volumes in an attempt to equalize the proportion of surface to volume of the different amounts of medium.'' By cross-infection made daily the bacterial food supply was maintained practically the same for all the experiments and, furthermore, this procedure rendered the bacterial products the same in the several volumes. Other investigators have demonstrated that ''the Infusoria in their ceaseless activity are continually voiding appreciable amounts of carbon dioxide and other products of metabolism,'' and so Woodruff concluded it to be highly probable that the Infusoria divided more rapidly in the higher volumes, because their own products of metabolism were less concentrated than in the smaller volumes.

To put this conclusion to a further test, a series of experiments was performed like those just mentioned, except that still another series of animals was carried which were changed to new medium every forty-eight hours and compared with those changed every twenty-four hours. From these experiments, Woodruff concluded that ''the gain of the series changed daily over those changed at forty-eight hour intervals is over eight per cent in the case of two drops and slightly over six per cent in the case of five, twenty, and forty drops,'' and so it was evident that the animals in the lowest volume of culture fluid divided much more slowly when the medium was changed at forty-eight-hour intervals.

Furthermore, Woodruff bred Paramecia in culture medium in which there had been a heavy growth of Paramecia, and found that it contained something toxic, since it decidedly depressed the division rate of the animals. Such an experiment seemed to be conclusive and apparently proved his postulate that the excretion products were the factors profoundly influencing the division rate of Protozoa bred in small volumes.

Finally, in 1913, Woodruff(9) determined that Paramecium aurelia in culture fluid in which an hypotrichous form had been growing multiplied faster than when bred in fresh uncontaminated media, but if the hypotrichs were bred in culture fluid which previously had supported a heavy growth of Paramecium aurelia, their division rate was slowed below that of the controls in uncontaminated culture medium. He interpreted the phenomena as being correlated with the faunal succession in large mixed hay-infusion cultures. Thus the products of certain Protozoa seem to be inimical to themselves, yet the same products may be beneficial or at least not inimical to another species, the entire action contributing toward a natural law of succession of various species of Protozoa as the dominant type in a culture.

Medrkiewiczowna(2), in 1921, studied the influence of the free surface of culture medium on the growth of cultures of Colpidium colpoda Ehr. and concluded that the amount of oxygen passing over the free surface was the principal factor in determining division rate. Another limiting factor of division rate under certain conditions was the accumulation of the products of metabolism. It was thought that this latter factor became dominant in cultures with very small free surface.

This generally accepted fact that infusorians tend to multiply more rapidly with increase of volume of culture medium was called into question by Robertson(4, 5, 6), in 1921 and later, who maintained in his studies on Enchelys farcimen Ehr. that cells mutually accelerated their division rate by the production of a chemical agent which he termed an 'autocatalyst,' and which was much more efficient in smaller volumes of fluid. His experiments indicated a slowing down and even cessation of the reproductive rate of Enchelys in large amounts of culture medium. The animals seemed to thrive better when large numbers were transferred to a given volume of culture fluid, and even when two animals were placed in the same drop of fluid, ''not twice, but from four to six or eight times as many individuals are produced in twenty-four hours as in culture which initially contained but a single individual.''

Furthermore, Robertson did not attribute the slowing or even cessation of division rate, of individuals isolated from old mass cultures to fresh culture fluid, to the retarding action of any of their products while in the original culture. This contention is supported by experiments in which it is apparently shown that, 1) even when the individuals are repeatedly washed in fluids of various kinds they do not recover reproductive capacity, and, 2) when an individual is isolated to a two- or a five-day infusorial culture filtrate, the division rate after twenty-four hours is practically the same as that of other cells isolated to hay infusion having a heavy growth of bacteria. But the results from a very few such isolations to the filtrate and to the infusion were cited and the animals employed apparently were not from pedigreed animals. If all the animals isolated to the fluids previously inhabited by Infusoria were of vigorous stock, it is extremely probable, even though the filtrates were contaminated with products of infusorian metabolism, that the cells isolated would not be affected in the earlier part of the twenty-four hours at least, and that the division rates noted were consummated before the healthy animals were affected sufficiently to permit depressant action to express itself in a lower division rate. He believed that the loss of division power in animals isolated from old mass cultures was due to the fact that the organisms had been subjected to ''a period of existence at a low nutritive level'' in the old cultures, since the bacteria in such cultures were just sufficiently numerous, probably, to keep the infusorians from starving.

As long ago as 1901, Wildiers(7) commented upon Liebig's inability to reproduce successfully Pasteur's experiments on yeast, noting that possibly one element entirely escaped Pasteur(3), who in 1869 published his observations that yeast needed only a mineral salt and a fermentable sugar for reproduction and fermentation. Wildiers commented: ''C'est quasi accidentellement qu'a surgi subitement devant nos yeux le fait nouveau qui explique à la fois les résultats de Liebig et de Pasteur; fait nouveau qui nous revèle en même temps que, contrairement à la loi de Pasteur, la levure est impuissante à édifier quoi que ce soit avec un milieu mineral et du sucre et qu'il faut un troisième élément qui a echappé à Pasteur.'' He demonstrated in his experiments that the unknown stimulative substance, or 'bios,' was not to be found in the yeast itself, but rather it was introduced into a yeast mass in some other substance, such as in Liebig's beef extract, in commercial peptone, or in wort. When he inoculated sterile media of sugar and mineral salts with small and large quantities of living yeast, only the large quantities survived. This, he believed, was because they brought with them a small amount of 'bios' from the wort on which they had been growing, and not because there were many cells there which stimulated each other by giving off some chemical substance. Wildiers states, ''D'ailleurs, la levure, en se multipliant et en fermentant, *ne produit pas de nouveau 'bios.'* Ce n'est donc pas un produit que la cellule de levure est capable de fabriquer. C'est un produit dont elle a absolument besoin pour vivre, mais qu'elle ne met pas en multiplication.'' In the case of Enchelys, Robertson found that it required only numbers to insure survival in larger amounts of medium, while one or two individuals inevitably died; that is, unlike the yeast, infusorians have the equivalent of this necessary 'bios' within themselves which is stimulated by a 'bios' from another body, such as an 'x-substance' from bacteria.

While the present study was in progress, Cutler and Crump(1) published the results of a series of experiments in isolating one or more cells to varying amounts of culture medium to test whether Robertson's allelocatalytic effect held true for Colpidium colpoda as well as for Enchelys farcimen.

Allelocatalysis was not found, but, on the contrary, if more than one animal was isolated to a slide, the division rates were not accelerated.

From this brief survey it is apparent that the results from the studies of the various investigators are widely divergent and in certain respects directly conflicting. Accordingly, the present series of experiments were undertaken to determine whether chemical products liberated by Infusoria retard or accelerate cell division.

I am indebted to Professor Woodruff for valuable advice and painstaking criticism throughout, and to the other members of the Yale University Zoölogy Staff for helpful suggestions.

MATERIALS AND METHODS

Four species of Protozoa were used, Paramecium aurelia, Paramecium caudatum, Pleurotricha lanceolata, and Stylonychia pustulata. A Paramecium aurelia was isolated on November 24, 1923, from a mass culture derived from Professor Woodruff's pedigree culture. A Paramecium caudatum and a Pleurotricha lanceolata were isolated on November 20, 1923, from a 'wild' laboratory culture. A Stylonychia pustulata was isolated February 7, 1924, from another 'wild' laboratory culture. Each animal was pedigreed for a short time before being used in an experiment.

For the experiments described under sections A, B, and C the culture medium used was hay infusion made up each evening as uniformly as possible, and when cool a pipette full of medium from that made up the day before was added. The whole was allowed to stand uncovered overnight and shaken before being used. Medium was made each forty-eight hours during the experiments presented under section D. The importance of the food factor is amply illustrated by certain incidental experiments in which the hay infusion was not inoculated with a pipette full of medium made up the day before. The results were extremely variable.

The organisms were bred synchronously in volumes of two, five, twenty, and forty drops of infusion so that temperature conditions were the same in all four volumes. Transference of individuals to sterile slides was effected by means of fine pipettes. After an individual had been transferred to a slide, the fluid carried over with it was reduced to a minimum by being drawn off with a pipette. After four slides with one animal each in the same type of drop had been so treated, the required number of fresh drops of hay infusion from the same flask was added by means of a pipette drawn out so that forty drops were practically equivalent to 1 cc. When two animals were transferred to a sterile slide, the required number of drops of culture medium were added while the animals were under observation with the dissecting microscope, so that both were known to be safely started in the new but similar environment. Isolations of as late division products as possible, as determined by size, were made at twenty-four-hour intervals.

Ordinary depression slides were used for carrying the animals in the two and five drops, and 50-mm. glasses for the forty drops. These were kept in moist chambers to prevent evaporation.

EXPERIMENTS

Any study of the factors governing the division rate of Infusoria must be concerned, first, in providing cells whose environmental history is known and, second, in controlling conditions as strictly as possible over a long period of time. In the present study these requirements, it is believed, have been fulfilled by the use of pedigree cultures of Infusoria for the determination of—

A) the influence of volume of culture medium on the rate of cell division;

B) the influence of cell proximity on the rate of cell division;

C) the factors concerned with a 'lag' period in the reproductive rate after the individual had been transferred from an old mass culture to a fresh medium;

D) finally, the effect, on the division rate, of supplying medium at twenty-four- and forty-eight-hour intervals, respectively.

A. The influence of volume of culture medium on the rate of reproduction

Twenty-seven five-day experiments were made to determine the average per diem division rate in different volumes of culture medium. For convenience in comparing results, as presented in table 1, the experiments are first grouped under species and second under periods 1, 2, and 3. A period includes three experiments with each species, or nine in all. Each experiment, which was of five days' duration, is here designated as either group I, II, or III, in order to indicate the fact that all the species in the same period and group were carried synchronously. Thus the division rates of the animals in any one period and group were influenced by the same general environmental conditions. The animals in each group in period 2 were descendants of those in the same groups, respectively, in period 1, and at the beginning of the period were five days further removed from a common ancestor than those in the beginning of the first period. The animals in period 3 were descendants of those in the same groups, respectively, in period 2, and at the beginning of the period were five days further removed from a common ancestor than those in the beginning of the second period, or ten days further removed than the more remote ancestors in the beginning of period 1. Each division rate, as given in table 1, is the average per diem division rate of four lines carried through a period of five days.

Those experiments for which the averages are shown in table 1, period 1, group I, extended from November 29, 1923, to December 3, 1923, while those in period 2, group I, extended from December 4 to December 8, 1923, and those in period 3, group I, extended from December 9 to December 13, 1923.

The common ancestor of the Paramecium aurelia, as well as that of the Paramecium caudatum employed in period 1, groups II and III, was isolated December 19th, from line 1 in the five-drop volume at the end of period 3, group I. The same holds true for Pleurotricha lanceolata, except that the common ancestor was derived from line 2 of the five-drop volume. The first periods of both the second and third groups

TABLE 1

The average daily division rates of three species of animals in twenty-seven experiments, each of five days' duration

GROUP	PERIOD 1				PERIOD 2				PERIOD 3			
	Drops				Drops				Drops			
	2	5	20	40	2	5	20	40	2	5	20	40
	Paramecium aurelia											
I	.95	1.35	1.75	1.55	.95	1.20	1.45	1.10	.85	1.15	1.30	1.50
II	.95	.95	1.05	.95	.90	.90	.75	1.00	.75	.75	.95	.85
III	.70	.85	1.00		.90	.85	.70		.70	.80	.70	
	Paramecium caudatum											
I	.75	.90	.90	1.20	.90	1.05	1.00	1.40	1.00	1.05	1.25	1.30
II	.95	.85	1.10	1.00	.85	.90	.95	.70	.80	1.05	1.05	.95
III	.90	.85	1.05		.75	.85	.95		.95	1.15	1.45	
	Pleurotricha lanceolata											
I	1.05	1.20	1.55	1.35	.75	1.10	1.45	1.65	.75	.85	1.40	1.60
II	.95	.95	1.30	1.35	1.25	1.30	1.45	1.45	1.40	1.65	1.75	2.05
III	1.00	1.25	1.35		.90	.75	1.05		1.45	1.70	2.00	

The average daily division rate of each species in all three groups and periods

	DROPS			
	2	5	20	40
P. aurelia.	.85	.97	1.07	1.15
P. caudatum.	.87	.95	1.07	1.09
P. lanceolata.	1.05	1.19	1.47	1.57
All three species.	.92	1.03	1.20	1.27

extended from December 29, 1923, to January 2, 1924; the second from January 3 to January 7, 1924, and the third from January 8 to January 12, 1924.

In the following discussion the average per diem division rates included under all three groups and all three species, in any one volume are compared with those of another volume. The division rates compared in the different volumes are those of animals carried synchronously.

Inspection of table 1 shows that in only four experiments out of a total of twenty-seven experiments did the animals divide more rapidly in an environment of two drops than in five drops. In four other experiments the division rates were the same in both volumes. In the remaining nineteen experiments the animals divided much more rapidly than those carried in two drops. This demonstrates that an environment of five drops is much more favorable for division rate for each of the three species tested than an environment of two drops.

Furthermore, in only four experiments out of a total of twenty-seven were the five drops more favorable for division than the twenty drops. The division rates in two experiments were the same in both volumes. The twenty drops supported a higher division rate in the remaining twenty-one experiments. Thus it is shown that an environment of twenty drops of culture medium is conducive to a higher division rate than an environment of five drops, and, indeed, by a greater margin than an environment of five drops is more favorable than two drops.

There was but a slight difference between the division rate of the animals in twenty and in forty drops. In eight experiments the twenty drops supports a division rate that is somewhat greater than that in forty drops, whereas the forty drops has a greater division rate in nine experiments. The division rates were identical in one experiment. The volumes of forty drops were carried in only eighteen experiments, i.e., in all experiments in groups I and II. The data show that the volume of culture medium for the maximum division rate is closely approached at twenty drops, and greater volumes have a progressively less accelerating effect.

Thus, all the experiments described point unequivocally to the conclusion that all three species of infusorians tested divide faster, within certain limits, in larger volumes of culture medium. And, moreover, further data pointing to the same conclusion will be presented incidental to the other series of experiments.

B. The effect of cell proximity on the rate of reproduction

The second series of experiments was planned to determine whether, as Robertson maintained in his studies on Enchelys, closely associated cells accelerate their own division rate by the production of a chemical agent or 'autocatalyst,' providing a stimulative 'x-substance' given off by bacteria is present. The series extended through February and March and consisted in a comparison of the division rate of one animal in a given volume with that of two related animals in the same volume of culture medium. The protozoon employed was Stylonychia pustulata from a pedigree culture.

In table 2 is shown the average per diem division rate for each of the sixteen five-day experiments, as well as for the contrasting sister controls. In the two-drop volume the one animal controls divided faster in all but experiments 100b, 102b, and 110 a, and in 105a where the division rates were the same. Where two individuals were bred together in the five-drop volume, the average per diem division rate was higher in only one instance, i.e., experiment 100a.

Further analysis of the data shows that the total average per diem division rate of the sixteen controls, as well as that of the two animals bred together, substantiate the results of the twenty-seven experiments performed with one animal in four different volumes of culture medium, to determine the influence of volume of culture medium on the rate of reproduction, described in section A, and so make a total of forty-three experiments pointing to the presence of some retarding influence on division rate in the smaller volumes.

In these experiments the daily division rate of the animals bred singly averaged 0.32 division higher than that of the animals carried together in the two-drop volume, and 0.27 division higher in the five-drop volume. There was, then, less difference in the division rates of the animal carried alone and the two carried together in the five-drop series than in the two-drop series. This indicates that in the larger volume the depressing effect of the presence of the two animals is less marked than in the smaller volume.

TABLE 2

The influence of cell proximity on the rate of reproduction of Infusoria

Experiments	THE AVERAGE PER DIEM DIVISION RATE OF SIXTEEN EXPERIMENTS AVERAGED FOR FIVE-DAY PERIODS															
	100		102		103	104	105		106	107		108		109		110
	a	b	a	b	a	a	a	b	a	a	b	a	b	a	b	a
	Two drops															
1 animal	2.40	1.35	2.25	1.60	1.45	1.75	1.05	1.25	1.40	1.00	.90	1.45	1.15	1.10	.75	.90
2 animals	1.70	1.50	1.60	1.70	1.20	1.25	1.05	.95	.80	.45	.60	.80	1.00	.50	.55	.95
	Five drops															
1 animal	2.35	1.95	2.30	2.45	1.90	2.15	1.70	1.85	1.55	1.55	1.50	1.65	1.70	1.40	1.45	1.55
2 animals	2.70	1.85	2.05	1.85	1.65	1.80	1.50	1.35	1.20	1.45	1.20	1.55	1.35	.85	.95	1.35

Summary

	NUMBER OF EXPERIMENTS SHOWING A GREATER DIVISION RATE IN	
	2 drops	5 drops
1 animal	12	15
2 animals	3	1

NOTE: Animals at the end of a 'b' experiment were five days further removed from a common ancestor than their ancestors at the end of an 'a' experiment. The division rates of all the animals in two drops in experiment 105 a are the same.

C. Repressed division rate

This series of experiments was conducted in order to secure data in regard to the temporary repression of division rate, or the so-called lag period, which has been described for certain Infusoria. The division rates of individuals transferred from an old 500-cc. mass culture to varying volumes of fresh medium have been contrasted with the division rates of pedigree animals transferred from a young mass culture to smaller volumes of fresh medium.

1. Animals transferred from old mass cultures. Experiment 200a, Paramecium aurelia. Sixteen individuals were isolated on February 11th from a mass culture which had been seeded December 17th with pedigree animals. Four of these sixteen animals were each placed in two drops of medium and the same number each in volumes of five, twenty, and forty drops, and transferred daily to fresh medium of the same volumes (compare table 4). At the end of twenty-four hours, only one animal out of the entire sixteen in the several volumes had divided even once and that in forty drops. Within the next twenty-four hours, nearly every slide held the products of two and three divisions. So a true lag period was definitely shown.

TABLE 3

Summary of the per diem division rates of animals bred singly and in pairs, as shown in table 2

VOLUME IN DROPS	1 ANIMAL	2 ANIMALS	DIFFERENCE IN DIVISION RATE
2	1.35	1.03	.32
5	1.81	1.54	.27
Difference in division rate	.46	.51	

Furthermore, at the end of the five-day period, the unrelated animals in each volume gave per diem division rates averaged for four lines, with the usual result of increased division rate in the larger volumes. In other words, it is a further demonstration of the truth of the conclusion reached in section A of this paper.

Experiment 201a, Paramecium aurelia. This experiment was a repetition of the previous experiment. The animals were isolated from the same culture and on the same day as in experiment 200a (compare table 4). A definite lag period was indicated in all four volumes, since there were no divisions during the first twenty-four hours, while there were averages of one or more divisions during the second day. This corroborates the results found in experiment 200a.

Experiment 202, Paramecium aurelia. Like 201a, this experiment was a duplicate of 200a in all respects except that the animals were isolated at a later date (February 18th), and again, a definite lag period was indicated during the first twenty-four hours (compare table 4). It was somewhat unfortunate that one line died the second day and another died during the fourth day in the two-drop volumes and that one line died during the fourth day in the five-drop volume, so that complete records for four lines in each volume at the end of five days were not available. However, the conclusion to be drawn from the data is certainly that a repressed division or lag occurred.

Experiment 204, Paramecium aurelia. Animals were isolated February 21st from the old mass culture started December 17, 1923. The average per diem division rate of the four lines in the two-drop volumes showed acceleration during the first twenty-four hours. The average division rate of the four lines in each of the other volumes is not indicative of a complete lag. Accordingly, the result of this experiment is at variance with the results of the three previous experiments in this series.

Experiments 212a, 213a, Paramecium aurelia. Twenty cubic centimeters was taken from a very rich culture of Paramecium aurelia which had been seeded on February 20th. To this was added 20 cc. of fresh hay infusion on March 27th, after which the mixture was allowed to stand undisturbed in a moist chamber until April 7th. Then isolations were made as in experiment 200a, except that volumes of eighty drops were used instead of forty drops. Clear-cut lag periods occurred in all volumes, except in two lines bred in the two-drop volumes in experiment 212a, which had a division rate of 1.00 each, while the other two lines showed at least a twenty-four-hour lag period. Thus an average division rate of 0.50 for the four lines in the two-drop volumes was the result. As a whole, these two experiments gave very similar results to those shown in the previous experiments in this series. Accordingly, the series, taken in its entirety, fairly justifies the conclusion that when Infusoria are transferred from one cul-

TABLE 4

The average daily division rates, of four lines in each volume, of animals transferred from old mass cultures to fresh medium

PARAMECIUM AURELIA

Exp.	200a			201a			202			204		212a			213a		
Days	1	2	5	1	2	5	1	2	5	1	2	1	2	5	1	2	5
Drops																	
2	.00	1.75	1.05	.00	1.00	1.13	.00	.33	.80	1.00	.75	.50	1.25	.90	.00	1.00	.80
5	.00	2.25	1.15	.00	1.75	1.20	.00	.66	.93	.25	1.50	.00	1.25	.70	.00	1.00	.75
20	.00	1.50	1.40	.00	1.00	1.25	.50	1.75	1.10	.50	1.75	.00	1.25	.85	.25	1.25	.80
40	.25	2.00	1.40	.00	1.75	1.26	.00	1.25	1.00	.75	1.75						
80												.00	2.00	1.20	.00	1.25	.80

	AVERAGES OF EXPERIMENTS NOT INCLUDING EXPERIMENT 204			AVERAGES OF ALL EXPERIMENTS	
Days	1	2	5	1	2
Drops					
2	.10	1.06	.93	.25	1.01
5	.00	1.38	.94	.04	1.40
20	.15	1.35	1.08	.20	1.41
40 and 80	.05	1.63	1.13	.16	1.65

NOTE: Under '1 day' is given the average division rate for the first twenty-four hours in the fresh medium. Under '2 days' is given the average division rate for the second twenty-four hours in the fresh medium. Under '5 days' is given the average per diem rate for the full five days.

ture fluid to a different culture fluid, e.g., from old to fresh culture fluid, their division rate is slower during the first twenty-four hours than thereafter; in short, there is a lag period.

2. Animals transferred from young mass culture. It has been shown that there is a tendency for a repression of division or lag, when animals are transferred to a medium markedly different from that to which they have been long subjected, and it is now important to determine if a similar lag occurs when pedigree animals are transferred from a large young mass culture to varying volumes of fresh medium. The two species of animals employed in these experiments were Paramecium aurelia and Stylonychia pustulata.

Experiments 205a, b, Paramecium aurelia. On March 4th, one animal was isolated to each of four lines in forty drops from a mass culture seeded February 20th. This culture at the time of isolation of the animals was not nearly so old as was the mass culture supplying the animals for the experiments shown in table 4. On March 5th, or twenty-four hours later, each of the animals in the four lines had divided once, giving an average of one division. On March 6th, the division rate had increased to an average of 1.75, indicating the presence of a slight lag period on March 5th, though not nearly so pronounced as in those lines derived from a much older mass culture. On March 5th, isolations were made to four lines in five drops, one animal being taken from each of the lines isolated to forty drops the day before. When the division rate of the animals in the five drops is compared with those in the forty drops, there is evident a marked drop in 205a in the division rate in the first twenty-four hours, but when contrasted with the division rate in the second twenty-four hours in the five drops, it is obvious that there was no lag period (compare table 5). The results from experiment 205b were very similar. The data from these two five-day experiments as a whole coincide with those of experiment 200b. Furthermore, these experiments were carried over a period of five days, which is long enough to indicate that the division

TABLE 5

	PARAMECIUM AURELIA (PA) AND STYLONYCHIA PUSTULATA (SP)																
Animal Exp.	PA 200 b		PA 205 a			PA 205 b			SP 206 a			SP 206 b			SP 207 a		SP 207 b	
Days		1	1	2	5	1	2	5	1	2	5	1	2	5	1	2	1	2
Drops																		
2[1]	.75	1.50																
5[1]	1.25	1.50	1.25	.75	.60	1.50	.75	.95	2.25	1.50	1.65	1.50	2.25	1.55	2.25	.50	2.00	.25
20																		
40		1.50	1.75	1.25	1.00	1.50	1.50	.95										
80									2.00	2.75	2.60	2.25	3.25	2.55	3.00	2.25	3.00	2.50

Animal Exp.	SP 208 a		SP 208 b		SP 209 a		SP 209 b		SP 210		SP 211			PA 212 b		PA 213 b	
Days	1	2	1	2	1	2	1	2	1	2	1	2	5	1	2	1	2
Drops																	
2									1.00	1.25	.75	1.50	.90				
5	1.75	.75	1.50	1.25	1.00	1.25	1.00	.75	1.00	1.25	1.50	2.25	1.45	1.75	.50	1.00	.25
20									2.00	2.25	1.00	2.25	1.70				
40																	
80	3.00	2.25	3.00	2.50	1.75	2.25	1.25	2.00	2.25	2.00	2.25	2.00	2.00	1.75	1.25	1.00	.75

[1] Pedigree control bred in 2 and 5 drops for five days.

For explanations see table 4.

rate of animals transferred to the five-drop volume soon becomes slower than that of the sister cells in the volumes from which they were transferred, even though it is not apparent during the first twenty-four hours. This is a further corroboration of the results already presented in section A.

Experiment 200b, Paramecium aurelia. This experiment was begun on February 17th, when each of the four lines in the forty-drop volumes of experiment 200a supplied one animal to each of four slides with two drops of culture medium, and one to each of four slides with five drops. The lines already in the forty drops were continued as controls. As a further control, the animals already in the two and five drops in experiment 200a were also continued.

It is evident from the data shown in table 5 that animals living for five days in two and five drops of culture medium, changed daily, divided more slowly than did the animals living in a similar small amount of fluid for only twenty-four hours. In neither the two nor the five drops was there any lag period as contrasted with the division rate in forty drops. Thus it is shown that transferring individuals from a volume of culture medium at least as great as is required for a maximum division rate to a small amount of the same kind of culture medium, but made twenty-four hours later, does not effect a significant lowering of the division rate during the first day. However, as is to be expected from our results already presented in regard to influence of the volume of culture medium, the division rate in the smaller volumes is faster at first than that of the animals bred in those volumes for a longer time.

Experiments 206a, b, Stylonychia pustulata. In all but the last two of the following experiments, Stylonychia pustulata was carried in place of Paramecium aurelia in order to determine the behavior of another species when subjected to similar treatment. The animals were isolated from a pedigree line in forty drops in experiment 107, placed in eighty drops of culture medium, and allowed to multiply for forty-eight hours. Then four lines in five drops were isolated for each of the two experiments, 206a and 206b. The former showed no lag period. The latter showed a lag period, which is not in accord with the previous results from the experiments in this series. It is highly probable that at the end of forty-eight hours the repressive factor in the parent culture medium was just beginning to be of a sufficient amount to check the metabolic rate of some of the cells, though not enough to affect all of them.

Experiments 207a, b, Stylonychia pustulata. These experiments were carried for three, instead of five, days, after being isolated at the end of twenty-four hours from a pedigree race in eighty drops. A lag period in the division rate did not occur during the first twenty-four-hour period after isolation to the lower volume.

Experiments 208a, b, and 209a, b, Stylonychia pustulata. The animals in these four experiments were isolated and carried as were the animals in experiment 207a, b. A lag period did not appear during the first twenty-four hours, except in experiment 209a. The reason for the failure of the latter experiment to agree with the previous ones is not apparent. However, it may be fairly concluded that the experiments, with this exception, show that, under like conditions, the behavior of the division rate of Stylonychia pustulata corresponds to that of Paramecium aurelia (compare table 5).

Experiments 210 and 211, Stylonychia pustulata. These experiments are somewhat unlike the others in this series, since the animals were taken from a large amount of culture fluid in which they had been living several days. The individuals were isolated on April 7th to four lines each in volumes of two, five, twenty, and forty drops from a 500-cc. mass culture started April 1, 1924. These two experiments are discussed here, since the culture from which the animals were taken was not 'old' in any sense.

Although the data do not show the complete twenty-four-hour lag period as in those animals taken from an old culture, yet there is a lag period of very short duration indicated in all volumes except the forty drops. This might be expected, inasmuch as the division products of the twenty pedigree animals seeded only seven days before in a large volume of medium could hardly have so modified the medium with the products of metabolism as to detrimentally influence the division rate of the animals. It is clear that the difference between the culture medium from which the animals were isolated and the new culture medium to which the isolations were made was not so great as an old culture medium would have been. These experiments are somewhat comparable to experiments 206a and b.

Experiments 212b and 213b, Paramecium aurelia. In these experiments Paramecium aurelia was again used. The individuals were isolated to four slides of five drops each from the twenty-four-hour-old cultures in eighty drops of experiment 212a and 213a, respectively, in the fourth day of their existence as pedigree lines. A lag period is not indicated during the first twenty-four hours. The lowered division rate of the animals in five drops during the second day obviously appeared too late to be interpreted as a lag, and probably affords an instance of the very marked influence of the effect of excretion products in a small volume. It will be noted, furthermore, that there is also a lowering of the rate of division of the animals in eighty drops during the second day as compared with their rate during the previous day.

D. The division rate of Infusoria bred forty-eight hours in the same medium, compared with the division rate of sister cells changed daily

As before stated, Woodruff(8) compared the division rate of Paramecium aurelia changed to new medium every forty-eight hours with that of others changed every twenty-four hours, and found that the animals remaining in small volumes a longer time divided more slowly than the ones changed twice as often. However, according to Robertson, Enchelys multiplies much slower in a fresh medium than a sister cell allowed to remain undisturbed in the old medium. This he attributes to the action of an 'autocatalyst,' diffused through the medium and manufactured by the cells themselves, in the presence of an 'x-substance' given off by bacteria.

Accordingly, experiments were made in an effort to clear up the question. The experiments were started by isolating one animal from a pedigree race to each volume of five drops of culture medium. Twenty-four hours later, one of the division products was allowed to remain in each volume of five drops of culture medium for twenty-four hours longer, or forty-eight hours in all (compare B, tables 6 and 7). A sister cell from each slide was transferred to fresh medium as the control. Any other division products were discarded.

TABLE 6

Paramecium aurelia

Slide no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	1
B	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1

TABLE 7

Stylonychia pustulata

Slide no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
A	3	1	1	3	3	3	3	3	3	1	3	1	3	2	3	3	3	3	3
B	0	0	0	1	1	0	1	1	0	0	0	1	1	3	1	1	1	1	died

A=division rate during the second twenty-four hours in fresh culture medium.

B=division rate of sister cells during the second twenty-four hours in the same culture fluid to which the parent cell was isolated forty-eight hours before.

The results of the experiments as presented in tables 6, 7, and 8[1] certainly indicate that Paramecium aurelia and Stylonychia pustulata reproduce faster in five drops of culture medium changed daily than sister cells allowed to remain forty-eight hours in the original culture medium. Accordingly, these results are not in agreement with those secured by Robertson with Enchelys. Furthermore, the results do not warrant the assumption of an autocatalyst. On the contrary, the depressed division rate is readily accounted for by the gradual accumulation of the normal excretory products of the cells.

[1] Experiments presented in table 8 were performed at Wesleyan University.

CONCLUSIONS

The experiments planned to determine if there is a relationship between division rate and volume of culture medium available for Infusoria afford conclusive evidence that larger volumes of culture medium, within certain limits, are more conducive to a more rapid division rate than are the smaller volumes which, on the contrary, show a very definite retarding influence on the division rate. This conclusion, which is in agreement with Woodruff's results, is further substantiated by the fact that when two cells are bred together in a given amount of culture fluid, they do not divide faster than one animal in a similar volume, but, as a matter of fact, divide much slower.

The experiments relative to a lag period in the division rate apparently substantiate Robertson's results, though it should be noted that if the division rate during the lag period be deducted from the subsequent division rate, then the animals isolated from the older cultures not only do not divide more slowly, but may even divide faster than those isolated from younger cultures. This accelerated division rate after a lag period is well shown in a comparison of experiments 200a and 201a, table 4, with experiments 205a and 205b, table 5. The data secured connect the lag with the degree of difference which exists between the medium to which an organism has been subjected and that new medium to which it has recently been transferred, since the lag is less prominent where the difference between the two media is not so great. But the explanation of the fact would seem to be that after recovering from the 'shock' of being placed in a new environment, the cells thereupon immediately expend their division energy, the expression of which had been temporarily suppressed by the new conditions to which they had suddenly been subjected. Another factor involved, as the experiments presented in this paper would indicate, is that the products of metabolism retard the division rate of animals in old cultures to such an extent that, when placed in a new environment, recovery to a more normal division rate is somewhat slow. In fact, it would seem that the products of metabolism in an old culture are chiefly responsible for a lag in the division rate of an animal transferred to an environment which is not contaminated with such products.

TABLE 8

Paramecium aurelia

																			Total divisions
Slide no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			
A	2	2	1	1	3	3	3	3	2	2	2	2	0	3	3	3			35
B	1	0	0	1	2	2	2	2	1	1	1	3	1	1	2	2			22
Slide no.	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	
A	3	1	1	3	2	2	0	1	1	2	1	1	2	2	1	1	2	1	27
B	2	0	1	2	3	1	1	0	0	1	0	1	2	1	0	0	0	1	16
Slide no.	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	
A	1	2	1	1	1	1	2	1	1	1	1	0	1	2	2	1	1	2	22
B	0	1	0	1	0	0	0	0	0	1	1	1	0	1	1	1	0	0	8
Slide no.	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	
A	2	1	1	1	1	2	1	1	1	2	1	2	2	1	2	1	1	2	25
B	0	0	0	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	9
Slide no.	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
A	2	1	1	1	2	2	2	2	2	1	1	2	0	1	1	0	1	1	23
B	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	0	14
Slide no.	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104			
A	2	1	2	2	1	1	0	1	2	1	2	1	1	2	0	1			19
B	1	1	1	1	1	0	1	0	1	1	1	1	0	0	1	0			11
																		Total divisions, A	151
																		B	80

Summary

$\begin{matrix}A\ 1\ \text{div.}\\B\ 0\ \text{div.}\end{matrix}$ appeared 28 times; $\begin{matrix}A\ 2\\B\ 1\end{matrix}$, 25 times; $\begin{matrix}A\ 3\\B\ 1\end{matrix}$, 1 time; $\begin{matrix}A\ 3\\B\ 2\end{matrix}$, 8 times; $\begin{matrix}A\ 2\\B\ 0\end{matrix}$, 9 times; $\begin{matrix}A\ 0\\B\ 1\end{matrix}$, 8 times; $\begin{matrix}A\ 2\\B\ 3\end{matrix}$, 2 times; $\begin{matrix}A\ 1\\B\ 1\end{matrix}$, 22 times; $\begin{matrix}A\ 2\\B\ 2\end{matrix}$, 1 time.

In the last noted twenty-three experiments where the number of divisions in A and B were the same at the end of twenty-four hours, it is significant that in eighteen of the experiments the divisions in A preceded those in B. In the remaining five experiments the division time was much more nearly the same.

The fact that the division rates of animals supplied with fresh culture fluid at twenty-four-hour intervals is higher than the division rates of animals bred under the same conditions, but with the medium changed at forty-eight-hour intervals, clearly indicates that the products of metabolism are, as Woodruff indicated, important factors in governing the metabolic rate of animals in old cultures and in very small volumes of fluid.

Broadly surveyed, the present study gives no evidence that the Infusoria studied elaborate a substance, called by Robertson an 'autocatalyst,' which in the presence of a bacterial 'x-substance' accelerates their cell division. The assumption of such an autocatalyst is clearly incompatible with the fact that animals divide more slowly in the smaller volume of culture medium as well as when two are associated in a given volume of medium.

SUMMARY

The results of the experiments performed show that:

1. The Infusoria studied tend to multiply faster in larger than in smaller volumes of culture medium.
2. The division rate of a single animal in a given volume of medium is faster than that of two animals in the same volume.
3. There is a repression of the division rate, or lag, shown by animals during the first twenty-four hours after being transferred from old cultures, in which the amount of the products of metabolism in the culture medium is probably very large, to fresh culture media. There is no apparent lag when animals are changed from young cultures to fresh medium.
4. Animals allowed to remain in the same medium for forty-eight hours show a much slower division rate than sister cells isolated to fresh medium at the end of twenty-four hours.
5. This is believed to indicate that products of metabolism excreted by the organisms repress their rate of division.

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SIDNEY IRVING SMITH AND HIS CONTRIBUTIONS TO ZOOLOGY.

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The death on May 6, 1926, brought to a close the life of Sidney Irving Smith, for thirty-one years professor of Comparative Anatomy at Yale University, and since 1906 professor-emeritus. Born in Norway, Maine, February 18, 1843, and receiving his early education at the Norway Liberal Institute, young Smith was brought to Yale in 1864 by his brother-in-law, Professor Addison E. Verrill, who had recently been appointed to the first chair of Zoology established at that University.

Before coming to Yale, Smith had already made an extensive collection of the insects of his native state. So carefully prepared and so accurately labelled was this collection that it was purchased by Louis Agassiz for the Museum of Comparative Zoology at Harvard College. Finding no similar collection at Yale, Smith spent parts of his vacations during many following years in making a new collection for Yale's Museum, and the Peabody Museum of Natural History at Yale now contains thousands of beautifully prepared specimens which testify to his zeal and skill as a collector and to his accuracy of identification.

In his senior year at Yale, Smith became a charter member of the Berzelius Society and for his essay on the Geographical Distribution of Animals was awarded the first of the prizes which have been given by that society. This paper was later published in the American Naturalist.[1]*

On graduating from Yale with the degree of Ph.B., in 1867 he was appointed assistant in Zoology in the Sheffield Scientific School, and with this institution he remained connected until his retirement from active service in 1906, being promoted to instructor in Comparative Anatomy in 1874 and to the professorship in Comparative Anatomy in 1875.

During all his summer vacations Smith was active in field work, gathering at such times material for his studies during the college year, for he was an indefatigable worker, for many years a bache-

* See the Bibliography at the end of this paper.

lor—his teaching and his research receiving his entire attention. His first official appointment outside the University was as zoologist on the United States Lake Survey in 1871. At this time a zoological reconnoissance of the deeper parts of Lake Superior was made and a number of new species of invertebrates discovered. The results of this work were published by him in several papers included in the Report of the Commissioner of Fish and Fisheries for 1872, part 2. One of these gives a summary of the invertebrate fauna of the lake, another discusses the food of fresh-water fishes, a third treats of the crustacean parasites of fresh-water fishes, including descriptions of new species, while a fourth consists of a synopsis of the higher fresh-water crustacea of the United States. Briefer papers on the same topics appeared in this Journal[2] and in the Canadian Naturalist.[3]

Portions of each summer from 1864 to 1870 were spent in assisting Professor Verrill on dredging expeditions in Long Island Sound and in the Bay of Fundy. Smith's attention was thus directed to the then almost virgin field of marine invertebrates, particularly the crustacea, and to this branch of zoology he devoted most of his life work, soon becoming the leading authority on the marine crustacea of the western Atlantic. In prosecution of these studies he joined the United States Coast Survey for work on St. George's Bank in 1872 and was a regular member of the scientific staff of the U. S. Fish Commission for the ensuing fifteen years.

During all this time he had charge of the crustacea taken in the dredgings, and thus made known to science a great number of new and interesting types, which have been of much value to later students in their interpretation of the relationships and evolutionary history of the crustacea.

The papers in which these new forms were described were published in this Journal,[4] in the Proceedings of the U. S. National Museum,[5] in the reports of the U. S. Commission of Fish and Fisheries,[6] in the Transactions of the Connecticut Academy of Arts and Sciences,[7] and in the Bulletin of the Museum of Comparative Zoology.[8] All of them are models of accuracy, faithfulness to details, skill in illustration, and discrimination in the interpretation of specific and generic relationships.

With characteristic generosity he presented all his cotypes and his extensive series of the various species in the collections to the Peabody Museum of Natural History at Yale and here they are available to other workers in that field of zoology.

Although Professor Smith's systematic work on the fresh-water and marine crustacea entitles him to a position in the front rank of American systematic zoologists, his studies on the life histories of the crustacea proved of more general interest. He was the first to interpret the successive stages in the larval life of the American lobster,[9] and his descriptions of the complicated metamorphoses which occur during the early life of other crustaceans[10] have found a wide application in interpretation of the relationships of the various groups.

For several years prior to 1874 he assisted Professor Verrill in the preparation of the classic Report on the Invertebrate Animals of Vineyard Sound; an ecological study that had no parallel in America for more than forty years.

While his later years were devoted entirely to the crustacea Professor Smith was a broadly trained naturalist of the old school, with a keen eye and an inquiring mind. An eager disciple of the then controversial Darwinian theory of evolution, he sought for verification of this hypothesis in all he saw about him. His first paper[11] was on the fertilization of orchids and his second on a new species of moth. He was much interested in insects, gathering extensive collections and reporting on the new and little known species which he found. In 1872-3 he served as State Entomologist of Connecticut and contributed to the reports of the Secretary of the Board of Agriculture for those years.

Smith was one of the founders of the Marine Biological Laboratory at Woods Hole, and one of the early members of its board of trustees. His last scientific work was the revision of the definitions in comparative anatomy for Webster's International Dictionary in 1890. Then in the prime of life he accepted ever increasing administrative duties in connection with the Sheffield Scientific School, and allowed himself to become burdened with committees to which he devoted himself with all the zeal which he had previously shown for science. In the sixteen years prior to his retirement he contributed much to the upbuilding of the University, but this was done, as is so often the case, at the sacrifice of his scientific career.

It is sad to record that during the last twenty years of his life Professor Smith was afflicted with hereditary glaucoma, which eventually led to total blindness. He bore this trouble with the patient resignation that is possible only in a person of his character and serene disposition. He was married in 1882, his wife dying in 1916. There were no children.

For thirty-nine years he was one of Yale's most inspiring teachers. He was one of the first zoologists in the country to put in practice Huxley's plan of intensive laboratory work in biology, comparative anatomy and embryology as the fundamental study for pre-medical students.

He was elected to membership in the National Academy of Science in 1884, and was one of the charter members of several of our biological societies. Yale conferred on him the honorary degree of M.A. in 1887.

His bibliography includes about seventy titles. Some of these papers are brief, others more extensive, but all are characterized by such thoroughness and faithfulness to detail that his work will stand the test of future generations, and will not have to be repeated.

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THE EFFECT UPON MAMMALIAN HEAT PRODUCTION OF FEEDING THYROID OF COLD-BLOODED VERTEBRATES [1]

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SIX FIGURES

Results obtained in a series of experiments by one of the writers on the factors involved in the metamorphosis of the frog tadpole clearly indicated that the physiologic responses of amphibians and mammals to thyroid administration depend upon different chemical groupings within the hormone (Swingle, '22, '23, '24). The essential fact brought to light in these experiments which has a bearing on the present work, was that substances which exert a thyroid-like effect when administered to amphibians may be without demonstrable effect upon mammals, the criterion for thyroid activity in amphibians being the metamorphic changes induced, and in mammals, the effect upon the basal metabolic rate. For example, certain iodized proteins, such as iodocasein, iodoserum globulin, iodoserum albumen, iodofibrin, and the amino-acid iodotyrosine, when fed or injected into thyroidectomized or hypophysectomized frog larvae, quickly induce metamorphosis, thus simulating a thyroid-like activity. These same substances are without effect upon amphibian transformation without iodine in the molecule, nor can other halogens, such as bromine, be substituted for the iodine, and the substances still retain their activity. Numerous workers have established

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[1] Part of the expenses of this investigation was defrayed by a grant from the Bache Fund of the National Academy of Sciences.

the fact that the iodized proteins and amino-acid mentioned produce no demonstrable effect upon the basal metabolic rate of normal mammals, nor do they alleviate the symptoms of myxoedema or cretinism (Oswald, '16–'17; Hellin, '97; Blum, '98; Strouse and Voegtlin, '09). The experiment of Kendall ('19) and Swingle, Helff, and Zwemer ('24) demonstrated beyond reasonable doubt that amphibian metamorphosis depends upon a peculiar organic iodine complex in the thyroid hormone, and that this complex, which is so active in the lower vertebrates, may be without effect upon the metabolic rate of mammals.

Injections of thyroxin into normal individuals exert a profound effect upon the basal metabolic rate. However, if the thyroxin is acetylated, all physiological activity is lost and the acetyl derivative can be injected into patients in enormous doses with impunity. One of the writers (K. M.) injected, at one dose, 29 mgm. of acetyl thyroxin into a normal subject, and was unable to detect any change in the metabolic rate over a period of several weeks. An equivalent dose of untreated thyroxin would raise the basal metabolism about 80 per cent, according to the quantitative studies made at the Mayo Clinic (Boothby, '23).

Despite its inability to affect the basal metabolic rate of mammals, acetyl thyroxin is highly active in inducing amphibian metamorphosis. The conclusion is evident that the metabolic responses of mammals and the metamorphic responses of amphibians to thyroid are hardly comparable, since they are apparently due to quite different chemical groupings within the hormone, i.e., the group responsible for metabolic change in mammals and the group which accelerates amphibian metamorphosis. In the case of the lower vertebrates, the iodine of the molecule is the active agent; in the case of mammals, it is iodine plus some other group, the presence of both groups being essential for the effect produced upon cellular oxidation.

Since the lower vertebrates, such as amphibians, are cold-blooded, and since it is only in warm-blooded forms that the unique effect upon heat production following injection of mammalian thyroid is elicited (Gayda, '22), the idea occurred to us that possibly the thyroid hormone of cold-blooded vertebrates might be deficient in, or even lack entirely, those chemical groupings concerned with the changes in heat production following thyroid injection in mammals. It was with this idea in mind that we performed the following experiments.

In order to test the idea, it was necessary to procure considerable quantities of thyroid material of cold-blooded vertebrates. Fish thyroids are not suitable on account of their small size and diffuse distribution, although the swordfish is said to have a large, compact gland. Alligator and sea-turtle glands were finally secured in sufficient quantities for work. The turtle thyroids were obtained through the courtesy of Mr. T. P. McTaggert, of the McTaggert Fish Products Company, New York City. The glands were removed by the writers from the freshly killed animals and cleared of all adhering tissue and desiccated. The glands were taken from large animals, sometimes weighing 500 pounds. The pure thyroid colloid can be obtained by puncturing the distended follicles and pressing out the viscid fluid.

The alligator material was obtained from freshly killed Florida alligators, the animals measuring from 4 to 12 feet, total length. For mammalian thyroid we employed Wyeth's (Philadelphia) commercial preparation of standard iodine content. In one experiment on rabbits, performed for us by Dr. David Marine, of New York, a preparation from Parke, Davis & Co. was used. The iodine content of all thyroid used is given in connection with the detailed data of the separate experiments.

We are greatly indebted to Doctor Marine for his generous cooperation in testing our reptile thyroid preparations upon rabbits by means of his modified Haldane respiration apparatus. Considerable data included in this paper were turned over to us by Doctor Marine. The writers are responsible for the interpretation.

The methods used for measuring the basal metabolic rate and for administering the thyroid were the same in all experiments. The desiccated thyroid was given by mouth in gelatin capsules in increasing doses for four days. The total amount given each individual was 80.4 grains (5.36 grams), the patient receiving 8 grains the first day, 16 the second, 24 the third, and 32 the fourth day. The morning of the first day the patient's basal metabolism was taken, using the Roth-Benedict closed-air method. The Tissot open-air method was employed in several experiments, but since the two methods check within an average of 1.5 per cent (3 per cent maximum) of each other, the choice of methods is immaterial. Following determination of the normal basal metabolic rate, the thyroid was administered, and the basal metabolism taken at definite intervals during the following two weeks.

The effect of commercial thyroid upon the basal metabolic rate of normal and hypothyroid individuals is well known, notably through the work of Boothby and his associates and numerous others. The workers at the Mayo Clinic have established a definite quantitative relationship between the amount of thyroid hormone administered and the effect upon basal metabolism. Despite the large amount of available data, we felt it necessary for comparison to measure the changes in basal metabolism of a normal individual following administration of mammalian thyroid of standard iodine content (0.2 per cent).

EXPERIMENT 1

Control subject fed mammalian thyroid

A normal adult male individual, age twenty-seven years, weight 57.4 kilos, was fed 80 grains (5.33 grams) of Wyeth's desiccated thyroid and the changes in basal metabolism noted. The iodine content of the glandular material was 0.2 of 1 per cent. The following abbreviated protocol gives the essential results of the test (protocol 1).

The data show a marked increase (27 per cent) in the basal metabolism beginning on the fourth day and reaching a maximum on the seventh and returning to normal on the eleventh. Subnormal levels were reached on the twelfth and fifteenth days. It will be noted that the pulse increased in proportion to the increase in metabolic rate. Marked subjective symptoms were noted, and on the sixth day following thyroid administration the subject was too ill to work. The symptoms and effects upon basal metabolism were practically identical with those reported by one of the writers (Swingle et al., '24) following an intravenous injection of 10 mgm. of thyroxin

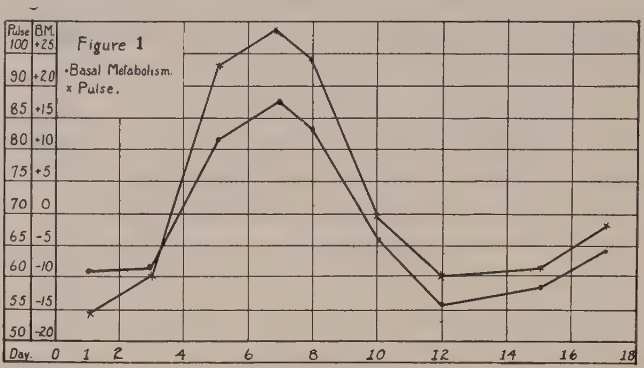
PROTOCOL 1

Control. Thyroid (Wyeth's)—effect on basal metabolism. Subject, G; age, 27

DATE	DAY	DOSE	PULSE	WEIGHT	BASAL METABOLISM
		grains			
1–31–26	1	8	54	57.4	— 8.0
2– 1	2	16			
2	3	24	60	57.5	— 7.4
3	4	32			
4	5	80	94	57.	+12.3
5	6				
6	7		98	56.	+17.5
7	8		94	56.	+13.
8	9				
9	10		66	56.	— 3.8
10	11				
11	12		60	57.5	—14.0
12	13				
13	14				
14	15		60	57.2	—12.5
15	16				
16	17		68	57.0	— 6.

2–2. No symptoms. Slight sore throat, but no pulse changes.
3. Complained of some depression and restlessness.
4. Complained of general malaise, in bed part of day.
5. General malaise, restless, palpitation, in bed.
6. The worst day. In bed. Symptoms as above.
7. Slightly better.
8. Much better, on duty.
9. No symptoms.
10. No symptoms.

into normal men. Figure 1 shows the change in the pulse rate and basal metabolism of this individual.



EXPERIMENT 2

Turtle thyroid, series A

Two normal young men were given 80.4 grains (5.36 grams) of desiccated sea-turtle thyroid over a period of four days. The iodine content of the material was 3.40 mgm. per gram of dried substance. It will be noted that the iodine content is distinctly higher than that of the mammalian thyroid used. The high iodine content of the thyroid of marine vertebrates

PROTOCOL 2

Thyroid (turtle)—effect upon basal metabolism. Subject, W.W.S.; age, 34

DATE	DAY	DOSE	PULSE	WEIGHT	BASAL METABOLISM
		grains			
10–16–25	1	8	64	62.5	— 9.9
	2	16			
	3	24			
	4	32			
	5	80			
	6				
10–23–25	7		66	61.6	— 9.1
10–29–25	13		60	61.4	—19.0

No symptoms save a little headache and general malaise on fourth and fifth days.

is due to the iodine of the sea-water and food. The protocol of one of the subjects of the experiment is given (protocol 2).

One individual showed practically no change in the basal metabolic rate following the thyroid administration. The other individual (K. M.) taking the turtle gland likewise showed little change, although both subjects reported slight headache and malaise on the fifth and sixth days. It is interesting to note that the basal metabolism fell to — 19 thirteen days after the beginning of the experiment on one subject. At the time this experiment was performed, sufficient turtle thyroid was not available to extend the observations. A few months later, however, several ounces of sea-turtle thyroid were obtained and four additional subjects were tested.

PROTOCOL 3

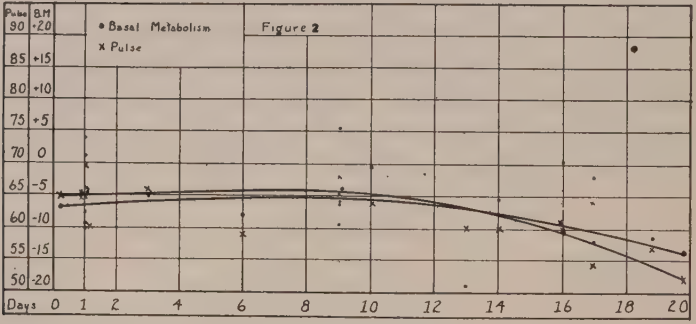
Thyroid (turtle)—effect on basal metabolism. Subject, C; age, 24

DATE	DAY	DOSE	PULSE	WEIGHT	BASAL METABOLISM
		grains			
1–30–26	1	8	60	65.2	— 5.4
31	2	16			
2– 1	3	24			
2	4	32			
3	5	80	64	63.5	— 4.5[1]
14	16		60	64	—10

[1] 2–3. Cold in head; feeling of general depression; no fever. No other symptoms.

Turtle thyroid, series B

The iodine content of the new material was 4.57 mgm. per gram of dried substance, whereas the first material collected about six months earlier was 3.40 mgm. This marked difference in iodine content is not to be attributed to seasonal differences, but to the fact that the second material was prepared somewhat differently, and consisted of pure colloid from the vesicles without admixture of tissue. Both lots of thyroid were from animals obtained from the same region of the Caribbean Sea. A typical protocol is given (protocol 3). All of the four subjects of the experiment showed a slight (3 per cent) but constant increase in basal metabolism at the end of the first week, returning to normal or below by the end of the second week. Figure 2 is a composite curve, showing the effect upon basal metabolism and pulse rate of feeding 5.36 grams of sea-turtle thyroid to six normal men. This curve includes the subjects taking sea-turtle thyroid A and B.



EXPERIMENT 3

Alligator thyroid

The amount of alligator thyroid at our disposal was limited, hence but a few subjects could be tested. The iodine content of the material was found to be 3.55 mgm. per gram of dried substance. Three normal young men, the writers and one other individual, were fed 80.4 grains (5.36 grams) of desiccated alligator thyroid within four days. Two protocols are given below for comparison (protocols 4 and 5).

Two individuals receiving alligator thyroid showed slight rises in the basal metabolism, whereas the third individual showed a sharp rise from — 8.3 to + 10.9 per cent. Examination of the protocol reveals the interesting fact that despite the rise in basal metabolism of about 19 per cent, the pulse rate changed but slightly and subjective symptoms were not noted. In fact, this subject was much surprised when told that his metabolic rate had increased considerably. Examination of the protocol of K. M. shows that the basal metabolism rate rose from —13.6 on the date of first feeding to —5.9 seven days later and then fell from —15 on the sixteenth day. The third individual reacted to the alligator thyroid but slightly. The basal metabolism rate rose from —15.2 on the date of first feeding to —11 on the fifth day and —9.4 on the eleventh day. It is obvious that —15.2 is abnormally low for this individual, and represents an abnormal depression on this date. Other tests on this individual

PROTOCOL 4

Thyroid (alligator)—effect upon basal metabolism. Subject, K.M.; age, 28

DATE	DAY	DOSE	PULSE	WEIGHT	BASAL METABOLISM
		grains			
12- 1-25	0	0	66	66.5	—11.4
12- 8-25	1	8	53	68	—13.6
	2	16			
	3	24			
	4	32			
	5	80			
12-15-25	7		54	67.	— 5.9[1]
12-24-25	16		54	66.5	—15.
1-12-26	35		58	67.4	—10.

[1] 12–15. Slight general malaise.
12–16. Slight general malaise.
12–17. O.K.

PROTOCOL 5

Thyroid (alligator)—effect upon basal metabolism. Subject, W.F.W.

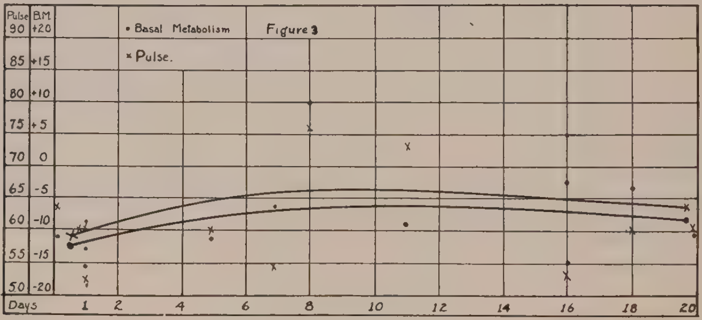
DATE	DAY	DOSE	PULSE	WEIGHT	BASAL METABOLISM
		grains			
11- 5-25	1	8	60	67.7	— 8.3
	2	16			
	3	24			
	4	32			
	5	80			
11-13	8		76	67.	+10.9
11-20	16		75	67.2	— 2.3

No symptoms.

(W. W. S.) show that the normal basal metabolism rate is around — 9, i.e., the lower limit of normal.

However, with the exception of one individual, the subjects of this experiment showed a moderate but fairly constant increase in heat production (6 per cent increase in basal metabolism) at the end of the first week, returning to normal or below at the end of the second week.

Figure 3 is a composite curve, showing the effect of alligator thyroid upon the pulse and basal metabolic rate of three normal adult men.



EXPERIMENT 4

Having determined the effect of reptile thyroid upon the basal metabolism of normal individuals, we next studied the changes produced by it on cases of hypothyroidism.

It is a common assumption that the first criterion for judging thyroid preparations is by their effect in relieving the symptoms of myxoedema and in raising the basal metabolic rate of such patients. The thyroid gland of myxoedematous individuals is hypo-active and the effect on heat production following administration of a substance in question is considered easier to judge.

We were unable to secure the cooperation of any cases of high-grade myxoedema, but were finally able to try the effect of reptile thyroid on three cases of incipient myxoedema with subnormal basal metabolism. These individuals, all of whom were females, had not previously received iodine or commercial thyroid. Each received 80.4 grains (5.36 grams) of desiccated turtle thyroid, the dosage being the same as in the previous experiments. Somewhat to our surprise, all of the subjects showed a marked increase in the basal metabolic rate. The increase was about 25 per cent. A typical protocol is given (protocol 6).

The fact that normal individuals reacted to equivalent doses of turtle thyroid by an average increase in basal metabolic rate of only 3 per cent, whereas the basal metabolic rate of cases of incipient myxoedema was 25 per cent, is interesting and suggestive. It is quite evident from this experiment that the turtle thyroid stimulated the hypo-active gland of the myxoedematous individual to secretory activity. There is a fallacy in the statement that the best method of testing the activity of thyroid of thyroxin preparations is by the effect produced upon the lowered metabolism of myxoedematous individuals, because it is not at all unusual for such individuals to react in a very striking manner to administration of iodine alone. Normal individuals usually show no response to iodine injection, but individuals exhibiting symptoms of hypothyroidism occasionally do so. For example, one of the writers (K. M.), in a series of unpublished experiments, has shown that the administration of Lugol's solution to such

PROTOCOL 6

Thyroid (turtle)—effect on basal metabolism. Subject, M.

DATE	DAY	DOSE	PULSE	WEIGHT	BASAL METABOLISM
		grains			
9-10-25	1	8	66		—21.
	2	16			
	3	24			
	4	32			
	5	80			
9-17-25	8		80		— 4.4
9-22-25	13		79		—11.6

Some general malaise on fourth and fifth days; no other symptoms.

individuals as employed in the present experiment may induce a sharp rise in the basal metabolic rate, equivalent to a 23 per cent increase. The effect on the basal metabolic rate in such cases is strikingly like that induced by thyroid feeding.

It is our belief that in cases of incipient myxoedema where the subnormal basal metabolism is the principal diagnostic feature, administration of substances containing moderate quantities of iodine may in certain individuals activate the hypofunctioning gland for an indefinite period. This seems to be true, especially in those cases where Lugol's solution induces a sharp rise in the basal metabolic rate. The ideal test for the activity of reptile thyroid upon the basal metabolic rate of myxoedematous individuals would be to receive the cooperation of an individual whose gland had been completely removed by operation, for in such a patient any question of activation of inactive thyroid tissue would be ruled out.

Figure 4

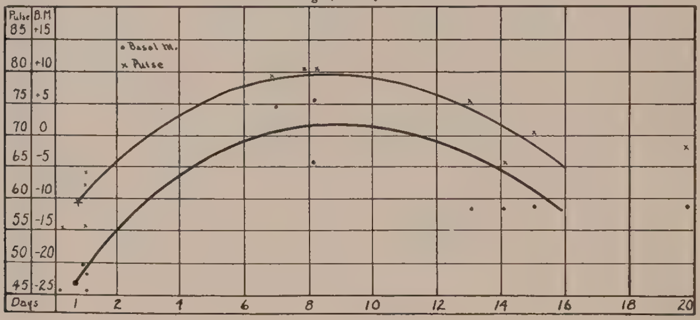
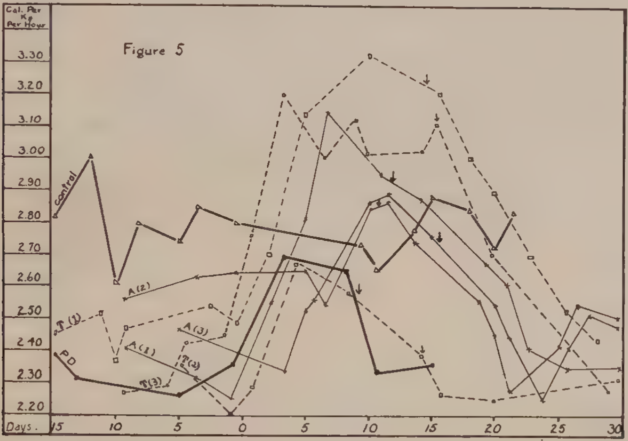


Figure 4 is a composite curve showing the effect of turtle thyroid upon the pulse and basal metabolism of three cases of incipient myxoedema. It is interesting to compare this curve with those shown in figures 1, 2, and 3.

EXPERIMENT 5

Effect of reptile thyroid upon the heat production of rabbits

Dr. David Marine, of the Montefiore Hospital, New York, became interested in our problem and generously offered to test the effects of reptile thyroid upon the heat production of rabbits by means of his modified Haldane open-air circuit apparatus for measuring the respiratory exchange of laboratory mammals (Marine, '21). This method as devised by Marine is very sensitive, and especially applicable to an experiment of this kind. Two kinds of controls were employed. The heat production of one normal adult rabbit of known age was determined at two-to-three-day intervals over a period of three weeks in order to obtain data as to the normal variation. This was found to be ± 0.15 calory per kilo per hour. Figure 5, control, shows the changes in heat production of this animal. The second type of control was a rabbit that received 200 mg. of Parke, Davis thyroid (iodine content, 0.2 per cent) every second day for five days, a total of 1000 mgm. being given. There resulted an increased heat production as a result of the thyroid administration, of 0.35 calory per kilo per hour. Figure 5 P.D. shows the changes in heat production of this animal.



The first set of experimental animals consisted of three rabbits. A1 received 200 mgm. of desiccated alligator thyroid (iodine content 3.55 mgm. per gram of dried substance) every second day until a total of 1400 mgm. had been given. There resulted an increase in heat production of 0.90 calory per kilo per hour. Animal A2 received 100 mgm. of alligator thyroid every second day until a total of 1400 mgm. had been given. There was an increased heat production of 0.25 calory per kilo per hour.

Rabbit A3 received 200 mgm. of alligator thyroid every second day until a total of 1400 mgm. had been given. There was an increase in heat production of 0.50 calory per kilo per hour.

PROTOCOL 7

Rabbit $_3$ *575. Male, born 11–28–24*

DATE 1925	ALLIGATOR THYROID FEEDINGS (BY MOUTH)	ROOM TEMPERATURE	DURATION	WEIGHT OF ANIMAL	TOTAL CO_2	TOTAL O_2	R. Q.	TOTAL CALORIES	CALORIES PER KILO	REMARKS
	mgm.		*hours*	*grams*	*grams*	*grams*			*per hour*	
11 – 16		22.0	2	2446	4.22	3.54	87	12.07	2.47	Quiet
11 – 18		21.4	2	2456	3.98	3.20	90	11.08	2.26	Quiet
11 – 21	200									
11 – 23	200	21.8	2	2440	3.91	3.36	85	11.39	2.33	Quiet
11 – 25	200	21.6	2	2398	4.16	3.54	85	12.11	2.53	Quiet
11 – 27	200	23.9	2	2391	4.11	3.64	82	12.31	2.57	Scratches occasionally
11 – 30	200	23.4	2	2410	4.66	4.05	84	13.70	2.84	Quiet, deep respirations, soft feces
12 – 2	200	20.9	2	2394	4.68	4.07	84	13.75	2.87	Quiet
12 – 4	200	21.3	2	2399	4.49	3.85	85	13.07	2.73	Quiet
12 – 7		22.4	2	2359	4.18	3.50	87	11.95	2.53	Quiet
12 – 9		22.0	2	2358	4.04	3.35	88	11.45	2.43	Quiet
12 – 11		19.8	2	2400	3.91	3.15	90	10.89	2.27	Quiet def.
12 – 14		19.6	2	2439	4.14	3.44	88	11.74	2.41	Quiet
12 – 16		21.4	2	2434	4.49	3.56	92	12.29	2.53	Quiet
12 – 18		18.8	2	2441	4.27	3.57	87	12.21	2.50	Quiet

Another series of animals, consisting of three rabbits, was fed sea-turtle thyroid and the effect upon heat production tested. Animal T1 received 200 mgm. of desiccated turtle thyroid (iodine content 3.40 mgm. per gram of dried substance) every second day until a total of 1200 mgm. had been fed. There resulted an increased heat production of 0.85 calory per kilo per hour. Animal T2 received 100 mgm. of turtle thyroid (iodine content 4.57 mgm.) every other day until 400 mgm. were fed. There was an increase in the heat production of 0.45 calory per kilo per hour. Animal T3 received 200 mgm. of turtle thyroid (iodine content 4.57 mgm.) every second day until 800 mgm. were fed. There was an increase in heat production of 0.75 calory per kilo per hour.

The effect upon heat production following administration of large quantities of reptilian thyroid to rabbits is striking and consistent. However, the dosage employed in these experiments is large. For example, in one experiment the amount of thyroid administered per kilo of body weight was over five times (5.8 times) that used on human subjects. In other words, an equal amount of thyroid per kilo of body weight fed to a human subject would necessitate giving a dose of about 31 grams. In the alligator experiment the dosage employed for one rabbit was 6.4 times that administered by us to human subjects (experiment 3). This would mean administering 34.3 grams of reptile thyroid within four days—an enormous dose. The results of this experiment are interesting when compared with that where Lugol's solution alone was employed (experiment 6).

The complete protocol of one rabbit is given on page 290 (protocol 7). Table 1, on page 292, gives in abbreviated form a summary of the entire experiment.

Because of the occasional striking rise in the basal metabolic rate following administration of Lugol's solution to cases of incipient myxoedema, we are inclined to attribute at least part of the increased metabolism following injection of reptile thyroid to the comparatively large quantities of iodine present. This point could have been checked by administering Lugol's to the same individuals after the basal metabolic rate had resumed its subnormal level. Unfortunately, we were unable to gain the cooperation of the patients for repeated tests. Since the rabbits tested by Marine's method reacted to the reptile thyroid by sharp rises in heat production following the oral administration of comparatively enormous doses, we suggested to Doctor Marine that he test the effect of Lugol's solution on his rabbits. He kindly consented to do this.

TABLE 1

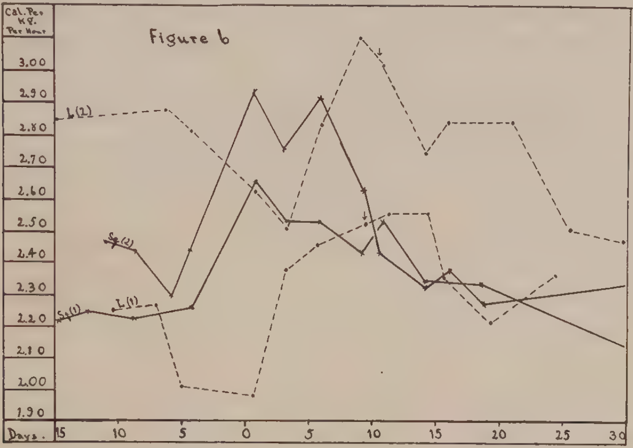
Summary of rabbit experiments. Metabolism in calories per kilogram per hour

	DAY OF EXPERIMENT	PARKE, DAVIS THYROID GLAND	ALLIGATOR GLAND			TURTLE GLAND	CONTROL ANIMAL	
		*200 mg.	*200 mg.	*100 mg.	*200 mg.	*200 mg.	DAY	
Before administration of gland	15	2.39				2.46	1	2.82
	14						2	
	13	2.31					3	
	12					2.53	4	3.09
	11						5	
	10					2.37	6	2.60
	9						7	
	8		2.41	2.57		2.47	8	2.80
	7						9	
	6						10	
	5	2.27			2.47		11	2.75
	4						12	
	3		2.31	2.63	2.26	2.51	13	2.86
	2						14	
	1	2.36	2.25	2.63		2.49	15	2.80
During administration of gland	1	*	*	*	*	*	16	
	2						17	
	3	*2.70	*2.53	*	*2.33	*2.70	18	
	4						19	
	5	*	*2.81	*2.64	*2.53	*3.15	20	
	6						21	
	7	*	*3.13	*2.54	*2.57		22	
	8	2.65				*	23	2.77
	9	*					24	
	10		*	*2.89	*2.84	*	25	
	11					*3.32	26	2.66
	12		*2.95	*2.82	*2.87		27	
	13						28	2.79
	14		*2.88	*2.92	*2.75		29	
	15						30	2.89
After administration of gland	1						31	
	2	2.32				3.21	32	
	3		2.68	2.53	2.53		33	2.88
	4					3.05	34	
	5		2.62	2.30	2.43		35	2.72
	6	2.36				2.90	36	
	7		2.40	2.48	2.27		37	2.84
	8							
	9					2.70		
	10		2.36	2.43	2.41			
	11							
	12				2.53			
	13					2.54		
	14		2.35		2.50	2.43		

EXPERIMENT 6

Effect of thyroxin and Lugol's solution on the heat production of rabbits

Four animals were employed in the experiment. One rabbit received an intravenous injection of 0.6128 mgm. of thyroxin (Squibb's). There was an increased heat production of 0.40 calory per hour per kilo. Figure 6, Sq. 1, shows the heat-production curve of this rabbit.



The second rabbit, Sq. 2, received an intravenous injection of 1.397 mgm. of thyroxin. This resulted in an increased heat production of 0.65 calory per kilo per hour (fig. 6, Sq. 2). The third animal received orally 0.075 cc. of Lugol's solution per day until 0.600 cc. were given. An increased heat production of 0.60 calory per hour per kilo body weight resulted (fig. 6, L1).

The fourth animal received 0.075 cc. of Lugol's solution per day until a total of 0.600 cc. were given. An increase in heat production of 0.60 calory per kilo per hour occurred (fig. 6, L2).

The experiment shows quite clearly that iodine itself in Lugol's solution, when administered to normal rabbits, may induce a sharp rise in the heat production.[2] It seems fair to conclude, in view of the data, that the iodine contained in Lugol's solution somehow stimulated the thyroids of these animals to increased activity. Certainly, in this experiment the indications are that Lugol's solution exerts its effect upon heat production solely through the mediation of the host's thyroid. The effect of Lugol's solution in rabbits is apparently in harmony with the fact that occasionally in myxedematous individuals a sharp rise in the basal metabolic rate follows administration of iodine. In both cases the thyroid gland of the experimental subject seems to be the agent directly responsible for the changes in heat production. It is unfortunate that it was impossible to employ more rabbits in this experiment. Two cases showed definite rises in heat production following Lugol's solution, but data on more animals are necessary before this point is clearly established.

DISCUSSION

The experimental work on the effect of thyroid, thyroxin, acetyl thyroxin, iodized proteins, and amino-acid upon amphibian metamorphosis (Swingle, '22, '23, '24) clearly demonstrates that the complete differentiation of these vertebrates is a problem in thyroid physiology, and is due to the iodine complex contained in the thyroid hormone. Other iodine-containing substances besides thyroid are capable of inducing effects similar to those produced by thyroid administration, so that in these forms the activity of thyroid upon differentiation is not unique. So far as is known, the sole function of the thyroid in cold-blooded vertebrates (e.g., amphibian larvae) is that of accelerating the differentiation incident to metamorphosis. It is well known (Allen, '17,–'18; Hoskins and Hoskins, '19; Swingle, '19–'22) that if the thyroid glands of frog larvae are extirpated the animals permanently retain their larval characters, but are otherwise normal. The writer has kept completely thyroidectomized frog and salamander larvae for over three years and the animals were normal at the end of this period except for their unusual size. In the absence of the thyroid, the larvae continue to grow, though eventually growth ceases when a certain maximum size is attained. Such animals can be metamorphosed completely within two weeks if fed thyroid or properly iodized substances. Growth ceases within a few days following thyroid administration, and differentiation begins. The evidence points to the conclusion that, in larval amphibians at any rate, rapid growth and rapid differentiation are mutually exclusive. When one process is taking place, the other is absent. Yet the thyroid gland is unnecessary for growth in Amphibia, but absolutely essential for differentiation. This is an interesting fact and is apparently contrary to conditions existing in mammals.

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[2] Two experiments of this kind are not sufficient to establish the important point that Lugol's solution raises metabolism in normal rabbits. The animals employed may have exhibited some thyroid hypertrophy and the iodine raised metabolism until the involution of the hypertrophy was complete.

There is another point of difference between amphibians and mammals in regard to thyroid physiology, namely, the unique action of the mammalian thyroid hormone upon heat production in warm-blooded vertebrates is extremely slight when such thyroid is fed or injected into cold-blooded forms like frogs (Gayda, '22). It is true that the metabolic rate of metamorphosing tadpoles, as gauged by oxygen consumption, is greatly increased during the period of transformation. But, as Helff ('24, '26) has demonstrated, such metabolic changes are incident to, and not a cause of, amphibian metamorphosis.

One of the writers (Swingle, '22, '23, '24) has shown that iodized substances which metamorphose thyroidless frog larvae are without effect upon mammalian metabolism (e.g., acetyl thyroxin). It seems clear that the thyroid gland has but one rôle in the lower vertebrates, and during the course of vertebrate evolution secondarily acquires a dual function. In the lower forms the iodine complex in the thyroid molecule functions as a regulator of metamorphic differentiation, whereas in the thyroid of the warm-blooded vertebrates another function is superimposed upon the primary differentiating function, i.e., that of producing a catalyst having to do with cellular oxidations and maintenance of a constant and high metabolic level. Apparently, during vertebrate phylogenesis certain chemical groups (e.g., those responsible for the metabolic changes following thyroid injection) were developed in warm-blooded forms, and function in connection with the iodine-containing complex responsible for differentiation, so that in mammals the two functions really become merged into one. If the thyroid is removed from immature mammals, not only does the individual fail to grow, but profound metabolic disturbances result.

The idea that a quantitative and possibly qualitative difference exists as regards the nature of the chemical groupings and constituents of the thyroid hormone of higher and lower vertebrates has considerable supporting evidence. For example, Kendall ('19) and Swingle, Helff, and Zwemer ('24) showed that thyroxin exerts a profound effect upon metabolism when intravenously injected, whereas the same substance when acetylated is inactive in mammals, but is highly active in amphibian larvae. Iodine was present in practically equal quantities and in the same combination in both compounds, but in the acetyl thyroxin certain other chemical groupings which ordinarily react with the iodine to produce the effects upon cellular oxidations were destroyed.

The results obtained in the present experiments likewise point to similar conclusions. The fact that 5.36 grams of sea-turtle thyroid, when fed to normal men, raised the basal metabolic rate but 3 per cent, whereas similar doses of mammalian thyroid (lower iodine content) raised the basal metabolic rate 27 per cent, is excellent evidence that reptile thyroid is less effective than that of mammal in changing the metabolic level. On the other hand, reptile and mammal thyroid are equally effective in accelerating metamorphic changes when fed to thyroidectomized amphibian larvae.

The results of the experiments where alligator thyroid was employed gave similar results, although the effect upon the basal metabolic rate of normal men was greater, causing a

6 per cent rise. We were able to test only three individuals, owing to paucity of material.

The work on rabbits seems at first sight contradictory to the theory of thyroid activity in vertebrates advanced here, since a sharp rise in heat production followed ingestion of the reptile glands. However, when the data of these experiments are analyzed it becomes evident that the dosage employed was extremely large, and, moreover, that the iodine content of the reptilian material was considerably higher than that of the control mammalian preparation. (Reptile thyroid lot A, 3.40; lot B, 4.57; alligator gland, 3.55 mgm., mammalian thyroid—Parke, Davis & Co., 0.2 per cent). It was previously stated that had we given reptilian thyroid to human subjects in doses (per kilo body weight) equivalent to that administered to rabbits A1 and A3, it would have necessitated giving 31 grams of thyroid within four days, or 7.75 grams a day. But despite the large dosage employed on the rabbits, there can be no question but that reptilian thyroid does contain small quantities of the chemical groupings within the hormone responsible for changes in the metabolic rate. According to our data, reptilian thyroid when fed to normal rabbits in doses of corresponding iodine content, is slightly more than half as active in inducing change in heat production following its administration as desiccated mammal thyroid.

There is a slight discrepancy between the results obtained on normal rabbits and those on normal human subjects. The experiments on the latter indicate that only a very small quantity of the substance concerned with metabolic changes is present in reptilian thyroid. On the other hand, the rabbit experiments indicate that this substance is present in reptilian thyroid in quantity about as great as exists in equal quantities of mammalian thyroid tissue.

An interesting point presents itself in connection with those experiments where reptilian thyroid raised the basal metabolic rate of cases of incipient myxoedema, and those in which Lugol's solution induced a rise in the heat production of rabbits (figs. 4 and 6). The commonly accepted criterion for judging the activity of any thyroid preparation is its effect upon the basal metabolic rate of mammals. However, it seems evident, from the data of these experiments, that the basal metabolism is not an absolute measure of the activity of any thyroid preparation, owing to the variable reactivity of the subject's own thyroid gland to iodine when such preparations are introduced into the organism. Such a test is absolutely reliable only when performed upon a completely thyroidectomized individual. Marine ('11) called attention to the possibility that iodine itself may be active in increasing metabolism, providing the individual retains sufficient active thyroid.

SUMMARY

The present experiments were designed to test the idea that possibly the thyroid hormone of cold-blooded vertebrates may be deficient in, or even lack entirely, those chemical groupings concerned with the changes in heat production following thyroid injection in warm-blooded forms. Since the lower vertebrates, such as amphibians, are cold-blooded, and since it is only in warm-blooded forms that the unique effect upon heat production following injection of mammal thyroid is elicited, the idea seemed worthy of exploitation.

The results obtained by feeding reptilian thyroid to mammals (man) indicate a quantitative difference in the thyroid hormone of cold- and warm-blooded vertebrates with reference to the chemical groupings in the molecule responsible for the changes in heat production in mammals following thyroid administration. Reptilian thyroid is less active in inducing such changes than is mammalian thyroid of equal iodine content.

Various problems of thyroid physiology suggested by results of the experiments are discussed in the text.

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3296

The Effect upon Amphibian Differentiation of Feeding Iodofibrin, Iodoedestin and Iodogliadin.

W. W. SWINGLE.

From the Zoological Laboratory, State University of Iowa.

Previous work by the writer[1] showed that the blood proteins, serum albumen and globulin, when fed to normal, thyroidectomized, or hypophysectomized frog larvae, exert no effect upon differentiation; but when properly iodized these same substances greatly accelerated the differentiation changes incident to metamorphosis. It will be recalled that thyroidectomized and hypophysectomized tadpoles do not metamorphose, but permanently retain their larval characters unless fed, injected, or transplanted with thyroid.

Iodofibrin.

Ten grams of pure fibrin from beef blood was pulverized and mixed with five grams of KI and two and a half grams of KIO_3. To this mixture was added a solution containing 2 cc. of concentrated H_2SO_4 and 300 cc. of water. The material was heated in a water bath at 90°—100° C. for 4 hours and stirred frequently. It was then filtered, washed first with water and then with dilute NH_4OH, followed by washing with water, alcohol and ether. The resulting product was iodofibrin.

Thirty completely hypophysectomized *Rana sylvatica* tadpoles averaging 30 mm. total length and showing no indications of metamorphosis were fed iodofibrin. Within 24 days, the entire culture, with the exception of 7 larvae which died at various stages of transformation, had differentiated to the stage where the animals had fore and hind limbs, frog mouth and marked tail resorption. All of the animals died at this stage of transformation.

A control culture of 30 hypophysectomized larvae fed non-iodized fibrin showed no metamorphic change at the end of 30 days feeding. The same experiment was repeated on completely thyroidectomized tadpoles with similar results.

Iodoedestin and Iodogliadin.

These 2 proteins were purchased in Germany and the method of iodization is unknown to the writer. Two series of 20 animals each, of thyroidectomized and hypophysectomized frog larvae, were fed iodoedestin and iodogliadin. Every third day a small quantity of algae were fed along with the iodized proteins. The hind limbs of the animals grew rapidly at first, but later, limb growth slowed down and metamorphic changes progressed slowly. It required 3 months of continuous feeding to bring the animals of these cultures to the same stage of metamorphosis as that which resulted from 20 days feeding with iodofibrin.

It was noted that considerable of the iodine split off from the edestin and gliadin after the powdered material had been in the water for an hour. Such was not the case with iodofibrin. It seems probable that the method of iodization employed has much to do with the activity of iodized proteins in accelerating amphibian transformation.

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[1] Swingle, W. W., *Biol. Bul.*, 1923, xlv, 229.

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3297

The Effect of Forcing Fluids, upon Survival after Bilateral Epinephrectomy.

E. L. COREY. (Introduced by W. W. Swingle.)
From the Osborn Zoological Laboratory, Yale University.

Previous work done by several investigators has shown that the administration of certain solutions may result in prolongation of life beyond the usual period in epinephrectomized animals. Stewart and Rogoff[1], working on dogs, report that intravenous injections of Ringer's Solution and dextrose prolonged life. Zwemer[2], working on cats, in this laboratory, was able to keep double operated animals alive for considerable periods by administering orally 5 per cent solution of glucose. This work suggested the possibility that forcing fluids containing various substances might prove beneficial in the treatment of adrenal insufficiency. Zwemer and Swingle have noted and commented upon the symptoms of dehydration which appear after bilateral epinephrectomy in cats, and, the fact that animals, presenting marked symptoms, eagerly drink large quantities of water up to within a few hours of death.

The procedure followed in the present work was to remove the left adrenal and after a lapse of 5 to 10 days extirpate the remaining glands. Untreated cats subjected to this procedure generally survived about 60 hours. The solutions were given by stomach tube, beginning immediately after the second operation. None of the animals employed were pregnant.

Ringer's Solution definitely prolonged life, the average survival period of animals receiving it being 174 hours. Doubling the percentage of the constituents of Ringer's Solution did not materially prolong the life span.

Sodium chloride given in 1 per cent solution prolonged life, the average life span of animals so treated being 149 hours.

Sodium bicarbonate likewise increased the life span, the average being 143.3 hours for animals treated with it.

Sodium acetate did not increase the life span, the average being 60 hours. The ineffectiveness of the acetate was probably due to its irritating effect upon the alimentary tract since the cats had difficulty in retaining it.

Solution A_1 containing magnesium lactate 0.9, strontium lactate 0.024, acid sodium phosphate 0.024 prolonged the survival period to an average of 150 hours.

On the other hand, Solution A_2 containing magnesium sulphate 0.9, strontium chloride 0.024, sodium sulphate 0.024 failed to prolong life, the average survival being 68.5 hours.

A 5 per cent solution of glucose increased the life span to an average of 180 hours.

It is not clear why forcing fluids should prolong the life of bilaterally epinephrectomized cats. The idea which most readily comes to mind in this connection is that the forcing of fluids and the consequent diuresis flushes some toxic substance out of the organism. Such treatment is merely palliative since all animals so treated eventually succumb with typical adrenal symptoms.

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[2] Zwemer, R. L., *Anat. Rec.*, 1925, xxxi, 338.

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3298

Blood Changes Following Bilateral Epinephrectomy in Cats.

W. W. SWINGLE.

From the Osborn Zoological Laboratory, Yale University.

During the last 3 years an intensive study of the functional significance of the suprarenal cortex has been carried on in this laboratory by the writer and his students. The present report is concerned with changes which occur in certain blood constituents following adrenal removal in cats.

It is well known that unilateral epinephrectomy is without demonstrable effect upon cats, hence animals subjected to such an operation were employed as controls. An interval of 5 to 7 days was allowed to lapse before the remaining adrenal was removed. Animals so treated generally survived loss of both adrenals for about 60 hours if untreated.

Phosphorus: The serum phosphorus shows a striking and consistent rise, following bilateral adrenal removal. The increase is considerable, especially when the animal is in the pre-coma or coma stage. Values as high as 15 to 16.5 mgm. per 100 cc. are not uncommon. The accumulation of phosphorus occurs simultaneously with the development of serious symptoms. Animals presenting slight symptoms do not show significant rises in phosphorus. The increase in serum phosphorus is apparently not due to increased blood concentration.

Calcium and *Sodium:* The results of repeated analyses are not of any significance. In general the calcium rises slightly and the sodium falls to about the same degree as that following bilateral epinephrectomy.

Non-protein nitrogen: The non-protein nitrogen always shows a sharp rise following adrenal removal, and occasionally the increase is very great. The average value for non-protein nitrogen, in unilateral operated cats, is between 42 to 47 mgm. per 100 cc., whereas, the average amount found in the blood of the double operated animals, presenting marked symptoms, is about 91 mgm. The non-protein nitrogen is highest when the animal is in or verging on coma. The increase becomes apparent within the first 24 hours after operation, and slowly increases, until the cat enters the pre-coma stage. Then a sharp rise occurs. Our highest figure for non-protein nitrogen was 170 mgm. per 100 cc.

Urea: The urea increases markedly after double operation, the normal value being 19 to 24 mgm.; whereas values ranging from 40 to 122 mgm. were observed in animals showing marked symptoms.

Protein and total nitrogen: These substances increase after the second operation. However, the increase in serum protein is not so great as we might expect, considering the apparent increase in blood concentration. The blood of animals in the pre-coma stage is concentrated, does not flow readily, clots rapidly, and yields small amounts of serum.

Blood Sugar: The blood sugar of normal cats ranges from 105 to 118 mgm. per 100 cc. Following bilateral operation the sugar drops markedly. The fall begins about 20 hours after the operation and continues slowly until death supervenes some 60 hours later. The blood sugar of cats verging on coma, 60 to 72 hours after operation, ranges from 60 to 40 mgm. per 100 cc. It was observed that the onset of symptoms of weakness and muscular asthenia in double operated cats coincides with the drop in blood sugar. Animals beginning to show muscular weakness and cerebellar symptoms, when walking, had blood sugar ranging from 80 to 75 mgm. As the sugar fell the weakness became more pronounced until finally, when coma developed, the blood sugar had fallen to 40 to 55 mgm. per 100 cc.

The pronounced hypoglycemia, following adrenal removal in cats, is not the cause of adrenal death, since animals injected with glucose eventually die with high blood sugar, although the life span of animals so treated is considerably prolonged. It is interesting in this connection to note that normal cats, injected with insulin, pass into coma and convulsions when the blood sugar reaches the same low level (40 to 55 mgm.) as that of bilaterally epinephrectomized cats in coma or convulsions. Our low figures for blood sugar were obtained from blood taken just before death.

The urine: The quantity of urine passed decreases sharply with the onset of serious symptoms. On the day when death occurs, or on the previous day, the animals void little urine. Some cats show almost complete suppression, others are able to pass small quantities until a few hours before death. Small amounts of albumin appear in the urine of animals showing marked symptoms.

Because of the phosphorus and nitrogen retention and the presence of albumin in the urine, the kidneys of double operated animals were studied histologically for degenerative changes. The results were disappointing in that, aside from hemorrhagic areas and congestion, clean cut pathological changes were absent. Nevertheless, the data indicate that the kidney is somewhat involved in the syndrome, which results in death following bilateral epinephrectomy. Experiments[1] on the acid-base equilibrium of epinephrectomized cats revealed the fact that one of the train of events leading to death from adrenal insufficiency is an uncompensated, non-volatile acidosis, which appears to be due to an increase of phosphoric and organic acids.

The complete data indicate that one of the causes of death following removal or destruction of the suprarenal cortex is acid intoxication due to accumulation and retention of the acid end products of normal metabolism. In the absence of the cortex the kidneys apparently fail to properly perform their acid eliminating function in relation to the neutrality regulation of the body. For some time, in some of our cases for 5 or 6 days, the respiratory mechanism is adequate to take care of the slowly advancing acid intoxication by throwing off volatile acid and the pH remains within the normal range. Finally there comes a time when the respiratory mechanism proves unequal to the task, and the acidosis becomes uncompensated, with falling pH and marked symptoms. The respiratory mechanism breaks down and death ensues from respiratory failure.

The type of acidosis, which develops following adrenal removal, is of a similar nature to the acidosis characteristic of the terminal stage of certain types of nephritis. It is suggested that the adrenal gland (cortex probably) supplies a hormone which is necessary for the maintenance of normal kidney function.

Complete papers presenting in detail the data summarized in this report will appear shortly in the American Journal of Physiology.

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3299

The Prevention of Tetany by the Oral Administration of Ammonium Chloride.

W. F. WENNER. (Introduced by W. W. Swingle.)
From the Osborn Zoological Laboratory, Yale University.

Previous experiments[1, 2] carried out on parathyroidectomized dogs have demonstrated the effectiveness of the lactates of strontium and magnesium in relieving tetany and in bringing about a cure. Experiments carried out last spring show that ammonium chloride also is an effective agent.

Parathyroidectomized dogs given a 5 per cent solution of ammonium chloride in 100 cc. doses twice daily, 3 to 4 hours before, and 5 to 8 hours after feeding, will retain the solution and may be kept free from tetany for long periods. One dog, reported here, never showed any symptoms of tetany save for a slight depression 4 days after operation. Once tetany appears, and the serum calcium falls below 7 mgm. per 100 cc., the ammonium chloride brings about a recovery within 2 hours, usually 1 hour and 15 minutes after administration. Examinations of the serum calcium at this time shows that the calcium has risen, on the average 2½ mgm. or to 8.5 mgm. per 100 cc., which is above the level at which tetany appears in dogs.

The experiments indicate clearly that ammonium chloride exerts a marked influence on tetany, probably more through its acidotic effect on the organism, than through an immediate effect on the serum calcium, although the rise in serum calcium may be sufficient to cause the disappearance of tetany symptoms.

Six dogs were parathyroidectomized. Dogs 4 and 6 were allowed to develop tetany; they recovered in 1 1/4 hours after receiving 100 cc. of 5 per cent solution NH_4Cl. The calcium of dog 6 rose from 6.6 mgm. to 10.1 mgm. per 100 cc. This was the greatest rise obtained for the 1 1/4-hour period. Dog 4 remained free from tetany for 4 days after the beginning of the ammonium chloride treatment, but died in tetany on the 6th day. Dog 6 recovered permanently. Animals 1, 2, 3 and 5 received ammonium chloride immediately after operation and twice daily thereafter. They showed marked tetany at different periods, followed by recovery after heavy doses of NH_4Cl. Dog 1 was depressed the 4th day after operation, but showed no other symptoms. Dogs 1, 2 and 5 recovered permanently. Dog 3 died of tetany and pneumonia on the 45th day.

After 30 to 40 days of ammonium chloride therapy the animals were placed on a meat diet and the ammonium chloride discontinued without ill-effects except in the case of animal 3.

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3300

The Acid-base Equilibrium of Epinephrectomized Cats.

W. W. SWINGLE AND ANNA J. EISENMANN.

From the Osborn Zoological Laboratory and Department Internal Medicine, Yale University, New Haven, Conn.

Because of striking changes in certain of the blood constituents, especially phosphorus, which follow adrenal extirpation in cats, and the fact that these animals present unusual changes in respiration a few hours preceding coma and death, it seemed probable that acid intoxication was one of the train of symptoms leading to death from epinephrectomy.

Unilaterally operated animals were used as controls. The amount of serum protein, total nitrogen, non-protein nitrogen, serum carbon dioxide, serum chloride, inorganic phosphorus and total base was determined. The pH was also determined.

The second adrenal was extirpated from 5 to 7 days after the removal of the first gland. The blood determinations were made again when the animals showed marked symptoms of adrenal insufficiency.

The blood was obtained with the usual precautions, and all the determinations were made accroding to standard procedure. The acid values of protein, bicarbonate, chloride, and phosphorus were calculated as described by Peters *et al.*[1] Total acid was taken to be the sum of all the determined acids. Organic acid was calculated by subtracting total acid from total base.

The results of the blood determinations showed that marked changes occurred in the blood following bilateral adrenal removal.

1. The tension of carbon-dioxide always dropped.
2. There was always a fall in pH.
3. The proteins rose in all except one case.

4. There was usually a marked rise in phosphorus.
5. The serum bicarbonate always diminished.
6. The total acid always diminished (*i. e.*, at the expense of bicarbonate).

Certain values showed variation in different individuals.

1. Changes in chloride concentration were variable.
2. Total base showed a striking drop in three animals.
3. Organic acid was not consistently affected, although it tended to be higher in the operated animals.

The fall, in pH, carbon-dioxide tension, serum bicarbonate and total acid, indicates that the bilaterally operated animals showing marked symptoms of adrenal insufficiency were suffering from an uncompensated non-volatile acidosis. This seems to be due to an increase of phosphoric and organic acids. In the early stages of adrenal insufficiency the acidosis is of the compensated type.

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3307

The Effect of Selection on the Rate of Reproduction of Paramecium Aurelia.

RAYMOND C. PARKER. (Introduced by L. L. Woodruff.)
From the Osborn Zoological Laboratory, Yale University, New Haven, Conn.

The work of Jennings[1] affords much evidence that, in *Paramecium,* morphological differentiations, which are hereditary, do not arise in a line reproducing vegetatively. As a result, selection is powerless to isolate hereditarily different strains among the descendants of a single cell. On the other hand, the same author[2] has shown that in the uniparental reproduction of a rhizopod, *Difflugia corona,* the number and length of the spines on the test, as well as other morphological characters, become differentiated in the descendants of a single individual; and that by selection it is possible to isolate many hereditarily different strains.

The effectiveness of selection in regard to what may be termed a physiological character has not been tested in *Paramecium,* but Middleton[3] has undertaken this in a study of the fission rate of another ciliate, *Stylonychia pustulata.* This author found that "by opposite selection, through more than 150 generations, of small individual variations occurring among the progeny of a single individual, it was possible to produce two sets differing hereditarily in rate of fission." The hereditary difference persisted through periods of balanced selection, and through conjugation, and disappeared only under the influence of reversed selection.

In view of the above results a series of experiments were carried on which involved the selection of a physiological character in *Paramecium.*

Although there is a remarkable synchronism of fission in closely related cells of a race of *P. aurelia,* under identical conditions, frequently one cell will divide slightly earlier than the others. This variation in the rate of division of individual cells is the physiological character which was selected in our experiments.

TABLE I.

A—Opposite Selection—200 days.

'Fast' lines	262 generations.
'Slow' lines	196 generations.

Difference in favor of fast, 66 ± 1.20.

A Opposite Selection		A_1 Balanced Selection		A_2 Reversed Selection	
225 days		225 days		225 days	
'F' lines	298	'F'-Bal. lines	295	'F'-Rev. lines	295
'S' lines	220	'S'-Bal. lines	219	'S'-Rev. lines	223
Difference	78 ±1.75	Difference	76 ±1.15	Difference	72 ±1.23
250 days		250 days		250 days	
'F' lines	336	'F'-Bal. lines	326	'F'-Rev. lines	327
'S' lines	236	'S'-Bal. lines	237	'S'-Rev. lines	244
Difference	100 ±1.70	Difference	89 ±1.89	Difference	83 ±2.94
275 days		275 days		275 days	
'F' lines	371	'F'-Bal. lines	361	'F'-Rev. lines	348
'S' lines	258	'S'-Bal. lines	261	'S'-Rev. lines	267
Difference	113 ±2.11	Difference	100 ±2.77	Difference	81 ±4.62
300 days		300 days		300 days	
'F' lines	401	'F'-Bal. lines	391	'F'-Rev. lines	375
'S' lines	275	'S'-Bal. lines	272	'S'-Rev. lines	286
Difference	126 ±3.27	Difference	119 ±2.16	Difference	89 ±3.84

The experiments summarized in Table I extended over a period of 300 days. From the progeny of a single animal eight lines were isolated; four of these lines were subjected to what might be referred to as a fast, or plus, selection over this period, the other four to slow, or minus, selection. At the end of 200 days the two sets showed a marked diversity in the rate of fission. The fast set attained an average of 262 generations, the slow set 196 generations. As indicated in the table, there was a very significant difference of 66±1.20 generations between the fast and slow lines at this time.

At the 200-day point two sets of sister animals were isolated from each of the A lines. One set of eight lines (A_1) was subjected to balanced selection (which amounts to no selection) for 100 days. That is, each plus selection was followed by a minus selection, at the next opportunity, and *vice versa*. Another set of eight lines (A_2) was isolated from the main diverse lines of A, and upon this was practiced reversed selection. That is, the fast lines were now subjected to slow or minus selection, the slow lines to fast or plus selection.

During this period of 100 days opposite selection was continued in the main (A) lines until we have a difference of 126±3.27 generations between the fast and the slow lines. Throughout this same period the lines subjected to balanced selection (A_1) maintained a difference in division rate of 119±2.16 generations, while the difference in the case of the reversed lines (A_2) had dropped to 89±3.84. Thus (taking into consideration the probable error) we find that this difference, which was cumulative, has persisted through the period of balanced selection, but tends to disappear under the influence of reversed selection. Selection has apparently been effective in establishing two diverse sets, differing in regard to the physiological character, division rate.

A more detailed analysis of these experiments will be presented in another paper. The effect of selection is now being studied in *Paramecium calkinsi,* a form which differs from *Paramecium aurelia,* in that endomixis has never been demonstrated, and apparently does not occur. The experiments are also being repeated on the hypotrich, *Stylonychia pustulata,* for the purpose of comparison with the two holotrichous forms mentioned above.

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